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Capillary Permeability in Skeletal Muscle

By

WILLIAM P. PAAKKE

Received 17 January 1977

Abstract

PAAKKE, W. P. *Capillary permeability in skeletal muscle* Acta physiol. scand. 1977 101: 1-14.

Capillary permeability in skeletal muscle was investigated by applying the single injection, external registration method in the arterioperfused cat gastrocnemius preparation. ^{51}Cr -EDTA (MW 341.2) and ^{59}Co -B12 (MW 1333.5) served as simultaneously injected indicators. At plasma flow of 7 ml/100 g tissue and an assumed capillary surface area of 70 cm²/g the permeability coefficients $P_d(^{51}\text{Cr}\text{-EDTA})$ and $P_d(^{59}\text{Co}\text{-B12})$ were $0.74 \cdot 10^{-4}$ cm/s and $0.43 \cdot 10^{-4}$ cm/s, respectively. However, correction for the recirculation of perfused capillaries yields $P_d(^{51}\text{Cr}\text{-EDTA}) = 1.5 \cdot 10^{-4}$ cm/s and $P_d(^{59}\text{Co}\text{-B12}) = 0.9 \cdot 10^{-4}$ cm/s. These results indicate that the permeability of muscle capillary for hydrophobic solutes is similar to that of cutaneous capillaries in other tissues. The Pappenheimer pore radius estimate of 30 Å, the Karnovsky 1 Å interendothelial slit width, and pinocytotic transport are defective in accounting for the results as ^{51}Cr -EDTA and ^{59}Co -B12 diffuse across the capillary membrane at rates proportional to their free diffusion coefficients in water ($\text{CDC}^{51}\text{Cr}\text{-EDTA}/\text{CDC}^{59}\text{Co}\text{-B12}) = \text{D}^{51}\text{Cr}\text{-EDTA}/\text{D}^{59}\text{Co}\text{-B12} = 1.79$). The transendothelial pore channel systems of fixed entities might be the primary for transcapillary exchange of hydrophobic molecules in this system alone could account for the experimental results. Only 1-2 open pores per μm capillary length are necessary to account for the observed permeabilities. For both indicators CDC was linear function of plasma flow and CDC increased 3-3.5 fold when plasma flow increased from 5 to 20 ml/100 g/min. Assuming constant permeability coefficients, the CDC increase with plasma flow effects an increase in capillary surface area indicating 3-3.5 fold recruitment of capillaries. A constant CDC of $\text{CDC}^{51}\text{Cr}\text{-EDTA}/\text{CDC}^{59}\text{Co}\text{-B12}$ within the perfusion range indicates that ^{51}Cr -EDTA and ^{59}Co -B12 can be employed at plasma flow 4 ml/100 g/min without risk of early back-diffusion resulting underestimation of permeability.

Pappenheimer, Renkin and Borrero (1951) studied permeabilities for hydrophilic solutes of different molecular weights by the biogravimetric osmotic transient method. From analysis of the results in accordance with the theory of restricted diffusion (Faxén 1922, Ferry 1936) an equivalent pore radius estimate of 30 Å was obtained. The general theory based on 30 Å pores (Pappenheimer 1943) was seriously questioned by Crone (1963 b) who found that sucrose and inulin permeated skeletal muscle capillaries at rates proportional to their free diffusion coefficients in water. Paaške (1977 a and b) used ^{51}Cr -ethylene-luminate-tetraacetate (^{51}Cr -EDTA) and ^{59}Co -cyanocobalamin (^{59}Co -B12) as indicators and obtained similar results for cutaneous and adipose tissues. These observations cannot be accounted for by the Pappenheimer pore radius dimension and it was doubted whether the

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- Supplementum 451. Measurement of Biogenic Amines Using Cation Exchange Chromatography and Fluorimetric Assay. By C. ATACK

blood vessels and nerves were ligated and cut, and all visible fat was removed from the muscle. The thigh muscles were separated from knee and distal part of femur. The crus was removed by disarticulation through the knee joint. The femur was plugged with cotton soaked in vaseline through a hole of 3 mm diameter which was drilled into the femur 2.0 cm proximal to the knee joint. A screw was inserted to plug the hole. The muscle was wrapped in moist gauze and covered with polyethylene sheets. The muscle was suspended between copper plates that were heated by a water thermostat to obtain constant muscle temperature of 37°C. During suspension the length of the muscle corresponded to its normal resting length. Femoral artery and vein and sciatic nerve were isolated at the lower 4/5 of the thigh. All side branches of the vessels were released and cut except for one small arterial side branch. The sciatic nerve was ligated and cut so that a 4 cm segment was obtained leading to the gastrocnemius muscle. The femoral vein was cannulated as close as possible to the muscle so as to allow collection of all effluent venous blood from the muscle. Blood was not recirculated. To compensate for blood loss from whole blood of 37°C was given through catheter introduced in the contralateral femoral vein. The contralateral femoral artery was catheterized to allow blood pressure measurements via Statham strain-gauge transducer on oscilloscope and Varian or Lofson meter. The animal was heparinized (1.250 IE/kg) and 150 µg (30 µg/ml) labelled ^{59}Co cobaltchloride was injected via the femoral artery catheter about 5 min prior to the indicator injection. I previously exposed the sciatic nerve was brought in contact with silver electrodes connected to a stimulator. The blood flow of the gastrocnemius muscle was adjusted by the stimulator to the desired constant blood flow value of an individual experiment. The width of the rectangular pulses was 0.1 ms and the voltage was 7 V. The pulse frequency was varied to obtain the desired blood flow (range 0.5–3 cm³/decade/h) (Kjellmer 1964). The needle (internal diameter 0.4 mm) of a micro-syringe was introduced into the small side branch of the femoral artery and the tip of the needle was positioned in the femoral artery. A one inch NaI (TI) scintillation detector or (PW 4111) Type A, Philips, (the Netherlands) was positioned above the muscle close to the surface. The detector was collimated to see the muscle, exclusively. Using the micro-syringe 3–7 µl bolus was injected into the femoral artery. The bolus contained 300 µCi ^{59}Co -cyanocobalamin (specific activity 150–300 µCi/µg; Boehringer-Mannheim, Germany) and 400 µCi ^{54}Co -methylcobalamin-tetracycline (specific activity 40 µCi/µg; A.O. Hoesche, Germany). The needle was withdrawn and removed immediately after the bolus had been given. Impulses recorded in the area of registration were routed from the scintillation detector to two microcomputer universal printing gamma spectrometers (Madsen & Sørensen, Denmark). The signals were pulse-height analysed with two individual windows of acceptance adjusted around the 720 MeV photopeak of ^{59}Co and the 0.122 MeV photopeak of ^{54}Co , respectively. Recorded counts including cross-talk are printed out (about five lines each) and were stored on paper tape as an interface to a computer and tape puncher (Type 4070, Facit AB, Sweden). Activity was recorded for 300 s. Effluent venous blood from the muscle was collected, and volume per time unit and haematocrit value were determined. The gastrocnemius muscle was removed and weighed. Activity remaining in the field was determined, corrected for cross-talk, and used as background. The data of the paper tape were fed into a program computer (Wang 60B, Wang Corp. USA) via an electronic high-speed reader (Type 4022, Facit AB, Sweden).

Calculations

The computer was programmed to cross-talk correct ^{59}Co activities, to subtract the cross-talk corrected background values, and to perform kinetic analysis. Cross-talk of ^{59}Co to the ^{54}Co channel was neglected (0.001). For each indicator the corrected count values were plotted as function of time in semi-logarithmic diagram by Type 602 plotter/puncher (Wang Corp. USA) connected to the computer. Kinetic analysis of the recorded curve function for each indicator was performed in accordance with the principles originally described by Sjöström (1970) using the computerized technique of Pankaj and Nielsen (1974). The count value of the injected dose of indicator was taken as the highest recorded value. Peak time is the time after the bolus injection at which the maximum count value occurred. The part of the curve recorded in the interval as indicated in Table II (Extrapol. start) was monoexponentially extrapolated to peak time using the "least square regression procedure". The numerical value of this function at peak time was subtracted from the originally recorded value to give the intravascular transit function. Intravascular mean transit time was computed as $\bar{t}(v)$ area/height (Zarler 1965). Plasma flow was calculated as $\bar{t}(p) = \bar{t}(v)/\bar{t}(p) \times 100$ (ml/100 g/min) (Kety 1951). $\bar{t}(p)$ denotes intravascular plasma flow as estimated at 0.03 (1–10%) (ml/g) here $\bar{t}(p)$ is haematocrit value (Sjöström and Tønnesen 1974). The value of the monoexponentially extrapolated curve at peak time fraction of maximum count value gives capillary extraction, E . The capillary diffusion cap

blood vessels and nerves were ligated and cut, and all visible fat was removed from the muscle. The thigh muscles were separated from knee and distal part of femur. The crus was removed by disarticulation through the knee joint. The femur was plugged with cotton soaked in vaseline through hole of 3 mm diameter which was drilled into the femur 2.0 cm proximal to the knee joint. A screw was inserted to plug the hole. The muscle was wrapped in moist gauze and covered with polyethylene sheets. The muscle was suspended above copper plate that was heated by water thermostat to obtain constant muscle temperature of 37°C. During suspension the length of the muscle corresponded to its normal resting length. Femoral artery and vein and sciatic nerve were isolated at the lower 4/5 of the thigh. All side branches of the vessels were isolated and cut except for one small arterial side branch. The sciatic nerve was ligated and cut so that 4-4 cm segment was obtained leading to the gastrocnemius muscle. The femoral vein was cannulated as close as possible to the muscle as to allow collection of all effluent venous blood from the muscle. Blood was not recirculated. Y components for blood loss fresh whole blood of 37°C was given through catheter positioned in the contralateral femoral vein. The contralateral femoral artery was catheterized in a slow blood pressure measurement can. Statham strain-gauge transducer on oscilloscope and Varian or Biomax writer. The animal was heparinized (1 250 IU/kg) and 150 μ g (30 μ g/ml) saline of ^{59}Fe was injected via the femoral artery catheter about 5 min prior to the endocrine injection. In exercise experiments the sciatic nerve was brought in contact with silver electrodes connected to stimulator. The blood flow of the gastrocnemius muscle was adjusted by the stimulator to the desired constant blood flow value of an individual experiment. The width of the rectangular pulse was 0.1 ms and the voltage was 7 V. The pulse frequency was varied to obtain the desired blood flow (range 0.5-5 capillaries/s) (Kjellner 1964). The needle (internal diameter 0.4 mm) of a micro-syringe was introduced into the small side branch of the femoral artery and the tip of the needle was positioned in the femoral artery. A one inch NaI (Tl) scintillation detector (FPH 4112, Type A, Philips, the Netherlands) was positioned above the muscle close to the surface. The detector was collimated to see the muscle, exclusively. Using the micro-syringe 5-7 μ l bolus was injected into the femoral artery. The bolus contained 300 μ Ci ^{59}Co -cyanocobalamin (specific activity 150-300 μ Ci/ μ g; the Radiochemical Centre, Amersham, Great Britain) and 400 μ Ci ^{54}Cr -ethylene-diamine-tetraacetate (specific activity 40 μ Ci/ μ g, AG Hoescht, Germany). The needle was withdrawn and removed immediately after the bolus had been given. Isotopes recorded in the area of registration were routed from the scintillation detector to two interconnected universal printing gamma spectrometers (Mediscan A/s, Denmark). The signals were pulse-height analysed with two individual windows of acceptance adjusted around the 0.520 MeV photopeak of ^{54}Cr and the 0.123 MeV photopeak of ^{59}Co , respectively. Recorded counts including cross-talk were printed out without time loss each and were stored on paper tape via an interface unit and tape reader (Type 4070, Fack AB, Sweden). Activity was recorded for 300. Effluent venous blood from the muscle was collected, and volume per time unit and hematocrit value were determined. The gastrocnemius muscle was removed and weighed. Activity remaining in the field was determined, corrected for cross-talk, and used as background. The data of the paper tape were fed into process computer (Heng 608, Wang Corp. USA) via an electronic high-speed reader (Type 4022, Fack AB, Sweden).

Calculations

The computer was programmed to cross-talk correct ^{59}Co activity, to subtract the cross-talk corrected background, and to perform kinetic analyses. Cross-talk of ^{59}Co to the ^{54}Cr channel was neglected (0.001). For each indicator the corrected count values were plotted as function of time in semi-logarithmic display by Type 602 plotter/printer (Wang Corp. USA) connected to the computer.

Kinetic analysis of the recorded curve function for each indicator was performed in accordance with the principles originally described by Sjöström (1970) using the computerized technique of Parks and Næfken (1974). The count value of the expected dose of indicator was taken as the highest recorded value. Peak time was the time after the bolus injection at which the maximum count value occurred. The part of the curve recorded in the interval as indicated in Table II (Extrapol interval) was monoexponentially extrapolated to peak time using the "least square" regression procedure. The numerical value of this function as solved for each was subtracted from the originally recorded value to give the intravascular curve function. Intravascular mean transit time was computed as $\bar{t}(iv)$ area height (Zierler 1965). Plasma flow was calculated as $\bar{t}(p) (1/\bar{t}(iv)) \times 100$ (ml/100 g min) (Kety 1951). λ denotes intravascular plasma volume back as estimated ± 0.03 (1-Hct) (ml/g) where Hct is hematocrit value (Sjöström and Törnqvist 1977) and plasma concentration. The slope of the monoexponentially extrapolated curve at peak time expressed in fraction of maximum count also gave capillary extraction, E . The capillary diffusion cap-

TABLE I Experimental results. $\text{CDC}^{(51)\text{Cr EDTA}}$ and $\text{CDC}^{(57)\text{Co-B12}}$ are presented in order of increasing plasma flow which was determined from direct measurement of venous outflow. The ratio $\text{CDC}^{(51)\text{Cr EDTA}}/\text{CDC}^{(57)\text{Co-B12}}$ was in mean 1.79 which is identical to the value of $D^{(51)\text{Cr EDTA}}/D^{(57)\text{Co-B12}}$, i.e. the ratio between the free diffusion coefficients in water at 37°C. Consequently Co-B12 is not subject to restricted diffusion as compared to $^{51}\text{Cr EDTA}$ in the bed of continuous capillaries of skeletal muscle. CDC for both indicators increase with increasing plasma flow indicating a 3-3.5 fold recruitment of capillaries.

Exp No	Plasma flow ml/100 g min	$\text{CDC}^{(51)\text{Cr EDTA}}$ ml/100 g min	$\text{CDC}^{(57)\text{Co-B12}}$ ml/100 g min	$\text{CDC}^{(51)\text{Cr EDTA}}/\text{CDC}^{(57)\text{Co-B12}}$
1	4.0	3.8	1.6	2.38
2	5.1	2.0	1.6	1.25
3	7.6	2.5	1.7	1.47
4	8.0	4.2	2.5	1.68
5	8.7	2.5	1.3	1.9
6	10.7	2.6	1.6	1.63
7	12.8	4.8	3.6	1.33
8	17.3	3.0	1.5	2.00
9	20.9	5.2	3.0	1.73
10	27.7	7.6	4.4	1.73
11	30.7	7.1	4.2	1.69
12	31.0	6.2	2.6	2.38
13	36.9	10.0	5.8	1.72
14	45.9	8.9	5.7	1.56
15	52.1	7.9	3.7	2.14
16	52.4	9.1	4.4	2.07
\bar{x}	23.2	5.5	3.1	1.79
S.E.	4.2	0.7	0.4	0.08

ity CDC , defined as the unidirectional flux of indicator across the capillary membrane per 100 g tissue per unit concentration difference across the capillary (Lassen and Trap-Jensen 1968 b) was calculated $\text{CDC} = -f(p) \cdot K \cdot \ln(1-E) \text{ (ml/100 g min)}$ where K is a constant for converting ml of plasma to ml of plasma ultrafiltrate. As K -values were used 0.89 for $^{51}\text{Cr EDTA}$ and 0.94 for $^{57}\text{Co-B12}$ (Lassen and Trap-Jensen 1968 b).

Results

Table I tabulates $\text{CDC}^{(51)\text{Cr EDTA}}$ and $\text{CDC}^{(57)\text{Co-B12}}$ together with the plasma flow as measured directly from venous outflow. Mean $\text{CDC}^{(51)\text{Cr EDTA}}$ was 5.5 ml/100 g min (S.E. 0.7 ml/100 g min, $n=16$), mean $\text{CDC}^{(57)\text{Co-B12}}$ was 3.1 ml/100 g min (S.E. 0.4 ml/100 g min), and mean $f(p)$ was 23.2 ml/100 g min (S.E. 4.2 ml/100 g min). A linear plot of $\text{CDC}^{(51)\text{Cr EDTA}}$ and $\text{CDC}^{(57)\text{Co-B12}}$ as functions of $f(p)$ is presented in Fig. 2. CDC for both indicators increases linearly with increasing $f(p)$. As calculated from the "least square" regression method, the function $\text{CDC}^{(51)\text{Cr EDTA}}$ vs $f(p)$ was satisfactorily described by the straight line equation $y = 2.12 + 0.144x$ ($r=0.90$, $p<0.001$). This indicates that $\text{CDC}^{(51)\text{Cr EDTA}}$ increases from 4.7 to 9.7 ml/100 g min within the plasma flow range from 4 to 52.4 ml/100 g min i.e., with a factor of 3.6. For $\text{CDC}^{(57)\text{Co-B12}}$ vs $f(p)$ $y = 1.43 + 0.071x$ ($r=0.78$, $p<0.001$). Thus, $\text{CDC}^{(57)\text{Co-B12}}$ increases from 1.7 to 5.2 ml/100 g min within the observed range, i.e. with a factor 3.1. The ratio $\text{CDC}^{(51)\text{Cr EDTA}}/\text{CDC}^{(57)\text{Co-B12}}$ was calculated for each experiment (Table I). Mean value was 1.79 (S.E. 0.08). Fig. 3 presents $\text{CDC}^{(51)\text{Cr EDTA}}/\text{CDC}^{(57)\text{Co-B12}}$ as function of $f(p)$ in a linear diagram. No

SKELETAL MUSCLE
Cr-EDTA & ^{57}Co -B12

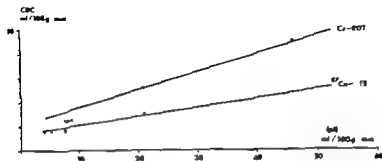


Fig. 2 The capillary diffusion capacity (CDC) of ^{51}Cr -ethylene-diaminetetraacetate (^{51}Cr -EDTA) and ^{57}Co -cyanocobalamin (^{57}Co -B12) as determined simultaneously by the single injection, external registration method at the plasma flow range 4-32.4 ml/100 g min. The function $\text{CDC}(\text{Cr-EDTA})$ vs. $\dot{Q}_p(\text{ml})$ obeys the straight line equation $y = 2.12 + 0.144x$. For $\text{CDC}(\text{Co-B12})$ vs. $\dot{Q}_p(\text{ml})$: $y = 1.43 + 0.071x$. Open circles: ^{51}Cr -EDTA, filled circles: ^{57}Co -B12.

systematic deviation could be ascertained from the ratio between the free diffusion coefficients for the two indicators in water at 37°C, $D(\text{Cr-EDTA})/D(\text{Co-B12}) = 1.79$ (solid line). By regression analysis $y = 1.66 - 0.006x$ ($r = 0.29$, $p > 0.1$, 95 per cent limits -0.20 to $+0.69$) indicating that significant correlation between the CDC ratio and plasma flow could not

SKELETAL MUSCLE
Cr-EDTA & ^{57}Co -B12

$\text{CDC}(\text{Cr-EDTA})/\text{CDC}(\text{Co-B12})$

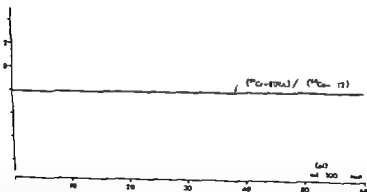


Fig. 3 The ratio $\text{CDC}(\text{Cr-EDTA})/\text{CDC}(\text{Co-B12})$ plotted as function of plasma flow (\dot{Q}_p). No systematic deviation from the 1.79 value of the ratio between $D(\text{Cr-EDTA})/D(\text{Co-B12})$, the ratio between the free diffusion coefficients at 37°C, could be ascertained and there is no reason to believe that back-diffusion of the indicators occurs from interstitium to plasma.

SKELETAL MUSCLE
 ^{51}Cr EDTA & ^{57}Co B12

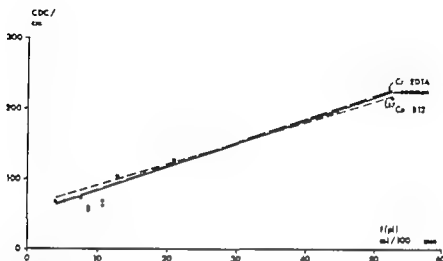


Fig. 4 The capillary diffusion capacity of each indicator was normalized with respect to the proper free diffusion coefficient in water at 37°C. Absence of restricted diffusion across the continuous capillary membranes of skeletal muscle is demonstrated by the close agreement of the regression lines. Consequently, transcapillary diffusion of the hydrophilic indicators takes place at rate constants proportional to the respective free diffusion coefficients. Open circles, ^{51}Cr EDTA; filled circles, ^{57}Co -B12.

be demonstrated. As restricted diffusion was not present for ^{57}Co -B12 as compared to ^{51}Cr EDTA ($\text{CDC}(^{51}\text{Cr EDTA})/\text{CDC}(^{57}\text{Co-B12}) - \text{D}(^{51}\text{Cr EDTA})/\text{D}(^{57}\text{Co-B12})$ see discussion) Fig. 4 was constructed for CDC values normalized with respect to the respective D-values. $\text{CDC}(^{51}\text{Cr EDTA})/\text{D}(^{51}\text{Cr EDTA})$ vs $f(\text{pl})$ yielded $y = 50.6 + 3.42x$ ($r = 0.90$, $p < 0.001$) and $\text{CDC}(^{57}\text{Co-B12})/\text{D}(^{57}\text{Co-B12})$ vs $f(\text{pl})$ gave $y = 61.1 + 3.02x$ ($r = 0.78$, $p < 0.001$). The common CDC/D function of $f(\text{pl})$ gave $y = 49.0 + 3.41x$ ($r = 0.84$, $p < 0.001$).

Mean capillary extraction of ^{51}Cr EDTA $E(^{51}\text{Cr EDTA})$ was 0.282 (S.E. 0.031) and mean $f(\text{pl})$ as calculated from the ^{51}Cr EDTA curve was 20.8 ml/100 g min (S.E. 3.5 ml/100 g min). Mean peak time for ^{51}Cr EDTA curves was 4 s (S.E. 1 s). Mean $E(^{57}\text{Co-B12})$ was 0.169 (S.E. 0.020) and a mean $f(\text{pl})$ of 23.9 ml/100 g min (S.E. 3.8 ml/100 g min) was calculated from ^{57}Co -B12 curves. Peak time for ^{57}Co -B12 was 4 s (S.E. 1 s) (Table II).

Significant differences could not be demonstrated between the directly measured plasma flow and plasma flow as determined by analysis of the indicator response curves ($p = 0.10$, Wilcoxon's rank sum test for paired observations). The directly measured $f(\text{pl})$ and the $f(\text{pl})$ as calculated by kinetic analysis did not indicate any significant increase in vascular volume from rest to heavy exercise.

The time interval chosen for monoexponential retroprojection of the later part of the externally recorded curve is listed for each experiment. Mean time for start of extrapolation was 8.97 $t_{(iv)}$ (S.E. 0.58 $t_{(iv)}$) where $t_{(iv)}$ was calculated from the venous outflow. Mean hematocrit value was 0.310 (S.E. 0.016) and the gastrocnemius muscle weighed in average 31.6 g (S.E. 0.9 g).

The experimental tracings of experiment No. 9 is presented in Figs. 5a and 5b.

TABLE II. Experimental results. Capillary extraction, E, and plasma flow (Qp), as calculated by kinetic analysis of the residue curves. For explanation of Peak time and Extrapolation interval, see text. Het denotes hematocrit values.

Exp No	⁵¹ Cr EDTA			⁵¹ Co-B12			Extrapol. interval	Het fraction	Muscle weight g
	E fraction	Q(p) ml/100 g min	Peak time	E fraction	Q(p) ml/100 g min	Peak time			
1	0.455	3.0	9	0.343	6.4	9	150-300	0.331	30.4
2	0.349	5.1	3	0.276	6.9	4	150-300	0.347	30.4
3	0.313	6.4	6	0.15	7.5	5	150-300	0.446	39.4
4	0.443	6.0	7	0.279	9.3	5	150-300	0.277	29.9
5	0.276	12.2	6	0.147	15.4	6	150-300	0.359	29.4
6	0.38	12.9	5	0.146	15.4	5	110-20	0.26	35.8
7	0.342	11.7	6	0.258	11.9	5	150-300	0.307	28.2
8	0.176	18	3	0.086	18.5	3	60-180	0.363	35.4
9	0.243	20.4	2	0.143	18.5	1	60-180	0.266	33.8
10	0.264	17.9	2	0.155	25.9		40-120	0.341	28.5
11	0.228	21	2	0.135	30.4	2	30-90	0.374	30.4
12	0.200	34.8	2	0.086	39.6	2	30-90	0.328	26.9
13	0.262	27.7		0.154	36.8	2	30-90	0.40	25.5
14	0.195	36.1	2	0.124	41.5		25-75	0.26	34.1
15	0.156	43.9	2	0.072	50.7	2	20-75	0.262	34.3
16	0.177	47.6	2	0.086	47.5	2	20-75	0.192	30.5
Σ	0.282	20.6	4	0.169	23.9	4		0.310	31.6
SE	0.031	5.5	1	0.020	3.8	1		0.016	0.9

Discussion

I. Methodological considerations

Application of the single injection, external counting method to a single inlet, single outlet system has been discussed elsewhere (Sejrsen 1970 Parske and Nielsen 1976) and only features of special significance for the present series will be considered.

As the residue curves follow a multiexponential function within the first hour of registration after the bolus injection (Lassen and Sejrsen 1971) the time interval of monoexponential extrapolation was chosen in order to obtain minimum effect of the bending of the curve fraction together with a reasonable number of observations (count values). Support of the validity of this procedure is indicated by agreement between directly measured blood flow from the venous outflow of the muscle and values obtained by kinetic analysis of the externally recorded response curves for each of the two indicators.

Expts. with bolus injection of intravascular indicators have shown that residue curves do not follow a monoexponential function as measured after bolus injection into the arterial bloodstream to the gastrocnemius muscle in cats (Sejrsen and Tønnesen 1972). The 1 per cent level of the height of the intravascular curve function was experimentally shown to be reached after a time period of approximately 9 s (iv). For this reason about 9 s (iv) was chosen as start time for monoexponential extrapolation (Table II and Results).

II. Permeability data

A. General considerations. The results indicate that CDC for both indicators increases linearly with plasma flow. Consequently it becomes essential that comparison between

SKELETAL MUSCLE
 ^{51}Cr -EDTA & ^{57}Co -B12

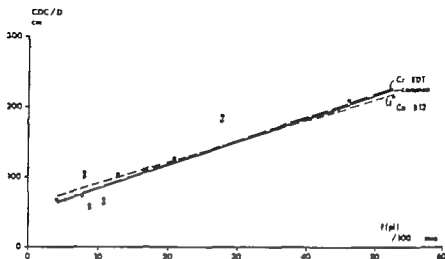


Fig. 4 The capillary diffusion capacity of each indicator was normalized with respect to the proper free diffusion coefficient in water at 37°C . Absence of restricted diffusion across the continuous capillary membranes of skeletal muscle is demonstrated by the close agreement of the regression lines. Consequently, transcapillary diffusion of the hydrophilic indicators takes place at rate constants proportional to the respective free diffusion coefficients. Open circles ^{51}Cr -EDTA, filled circles ^{57}Co -B12.

be demonstrated. As restricted diffusion was not present for ^{57}Co -B12 as compared to

^{51}Cr -EDTA ($\text{CDC}(^{51}\text{Cr}\text{-EDTA})/\text{CDC}(^{57}\text{Co}\text{-B12}) - \text{D}(^{51}\text{Cr}\text{-EDTA})/\text{D}(^{57}\text{Co}\text{-B12})$ see discussion) Fig. 4 was constructed for CDC values normalized with respect to the respective D-values. $\text{CDC}(^{51}\text{Cr}\text{-EDTA})/\text{D}(^{51}\text{Cr}\text{-EDTA})$ vs $f(\text{pl})$ yielded $y = 50.6 + 3.42x$ ($r = 0.90$, $p < 0.001$) and $\text{CDC}(^{57}\text{Co}\text{-B12})/\text{D}(^{57}\text{Co}\text{-B12})$ vs $f(\text{pl})$ gave $y = 61.1 + 3.02x$ ($r = 0.78$, $p < 0.001$). The common CDC/D function of $f(\text{pl})$ gave $y = 49.0 + 3.41x$ ($r = 0.84$, $p < 0.001$).

Mean capillary extraction of ^{51}Cr -EDTA $E(^{51}\text{Cr}\text{-EDTA})$ was 0.282 (S.E. 0.031) and mean $f(\text{pl})$ as calculated from the ^{51}Cr -EDTA curve was 20.6 ml/100 g min (S.E. 3.5 ml/100 g min). Mean peak time for ^{51}Cr -EDTA curves was 4 s (S.E. 1 s). Mean $E(^{57}\text{Co}\text{-B12})$ was 0.169 (S.E. 0.020) and a mean $f(\text{pl})$ of 23.9 ml/100 g min (S.E. 3.8 ml/100 g min) was calculated from ^{57}Co -B12 curves. Peak time for ^{57}Co -B12 was 4 s (S.E. 1 s) (Table II).

Significant differences could not be demonstrated between the directly measured plasma flow and plasma flow as determined by analysis of the indicator response curves ($p > 0.10$, Wilcoxon's rank sum test for paired observations). The directly measured $f(\text{pl})$ and the $f(\text{pl})$ as calculated by kinetic analysis did not indicate any significant increase in vascular volume from rest to heavy exercise.

The time interval chosen for monoexponential retroprojection of the later part of the externally recorded curve is listed for each expt. Mean time for start of extrapolation was 8.97 i(iv) (S.E. 0.58 i(iv)) where i(iv) was calculated from the venous outflow. Mean hematocrit value was 0.310 (S.E. 0.016) and the gastrocnemius muscle weighed in average 31.6 g (S.E. 0.9 g).

The experimental tracings of expt. No. 9 is presented in Figs. 5a and b.

TABLE II. Experimental results. Capillary extraction, E , and plasma flow $f(p)$, as calculated by kinetic analysis of the residue curves. For explanation of Peak time and Extrapolation interval, see text. Hct denotes hematocrit, also.

Exp No	$^{51}\text{Cr-EDTA}$			$^{51}\text{Cr-BI}$			Extrapol. interval	Hct fraction	Muscle right %
	E fraction	$f(p)$ ml/100 g min	Peak time	E fraction	$f(p)$ ml/100 g min	Peak time			
1	0.855	5.0	9	0.343	6.4	9	190-300	0.331	30.4
2	0.349	6.1	3	0.276	6.9	4	190-300	0.347	12.6
3	0.313	6.4	6	0.215	7.5	5	190-300	0.446	39.4
4	0.443	8.0	7	0.779	9.3	5	190-300	0.277	29.9
5	0.776	12.2	6	0.147	15.4	6	190-300	0.359	79.4
6	0.238	12.9	5	0.146	15.4	5	110-150	0.262	35.8
7	0.342	11.7	6	0.238	11.9	5	140-300	0.307	28.2
8	0.176	18.2	3	0.084	18.5	3	60-180	0.363	35.4
9	0.241	20.4	2	0.143	18.5	1	60-180	0.266	35.8
10	0.264	17.9		0.153	25.9	2	40-170	0.341	28.3
11	0.228	21.2	2	0.135	30.4		30-90	0.374	30.4
12	0.200	36.8	2	0.086	39.6	2	30-90	0.328	26.9
13	0.262	27.7	2	0.134	36.8	2	30-90	0.240	25.5
14	0.195	36.1	2	0.14	41.5	2	25-75	0.762	34.1
15	0.156	43.9	2	0.072	30.7	2	20-75	0.262	34.3
16	0.177	47.6	2	0.086	47.5	2	20-75	0.192	30.5
	0.282	20.6	4	0.169	23.9	4		0.310	31.6
SE	0.031	3.5	1	0.020	3.8	1		0.016	0.5

Discussion

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Expts. with bolus injection of intravascular indicators have shown that residue curves do not follow monoexponential function as measured after bolus injection into the arterial bloodstream to the gastrocnemius muscle in cats (Sejnen and Tonnesen 1972). The 1 per cent level of the height of the intravascular curve function was experimentally shown to be reached after a time period of approximately 9 (iv). For this reason about 9 (iv) was chosen as start time for monoexponential extrapolation (Table II and Results).

II. Permeability data

A. General considerations. The results indicate that CDC for both indicators increases linearly with plasma flow. Consequently it becomes essential that comparison between

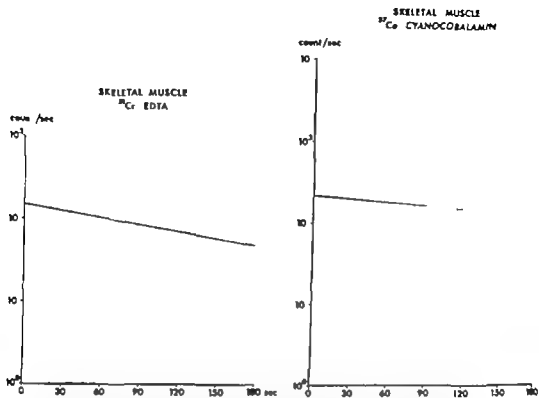


Fig. 5. Fig. 5a presents the computer printout of the recorded count rates for ^{51}Cr EDTA corrected for background activity (9 cps) plotted as a function of time in a semilogarithmic diagram (dots) (E. pt. No. 9). At time zero the bolus injection was given. A maximum count value of 617 cps was reached at 2nd s (post time). The part of the curve recorded in the time interval 60 s–180 s obeyed the equation $\text{cps}(t) = 151 \exp(-0.0063 t)$ where t is time in s. The standard deviation on the slope $SD(k)$, was 0.0003 s^{-1} . At peak time (2nd s) a value of 150 was obtained. Capillary extraction $E(^{51}\text{Cr EDTA})$, was $150/617 = 0.43$. Plasma flow as measured directly was $20.9 \text{ ml}/100 \text{ g min}$ and $\text{CDC}(^{51}\text{Cr EDTA}) = 20.9 \cdot 0.89 \ln(1.04) = 18.1 \text{ ml}/100 \text{ g min} = 5.2 \text{ ml}/100 \text{ g min}$. As the mean transit time of $^{51}\text{Cr EDTA}$ plasma was 0.108 sec (crosses denote the vascular transit) the plasma flow could be calculated from $f(\text{pl}) = (1/0.108) \cdot (1.076) = 0.03 \cdot 100 \text{ (ml}/100 \text{ g min}) = 3.0 \text{ ml}/100 \text{ g min}$.

The simultaneously recorded $^{57}\text{Co-B12}$ traces given in Fig. 5b, where count values corrected for cross-talk and background activity are plotted as function of time in a semilogarithmic diagram. Cross talk correction factor from ^{51}Cr to ^{57}Co was 0.48. Background value for $^{57}\text{Co-B12}$ was 22 cps. Maximum count value, 1510 cps, was reached at 1st s. The extrapolation slope was given by $\text{cps}(t) = 215 \exp(-0.0030 t)$ with $SD(k) = 0.0003 \text{ s}^{-1}$. $E(^{57}\text{Co-B12})$ was $151/510 = 0.143$ g using $\text{CDC}(^{57}\text{Co-B12}) = 3.0 \text{ ml}/100 \text{ g min}$. As determined from kinetic analysis of the $^{57}\text{Co-B12}$ curve plasma flow was $18.1 \text{ ml}/100 \text{ g min}$. The ratio $\text{CDC}(^{51}\text{Cr EDTA})/\text{CDC}(^{57}\text{Co-B12})$ was 1.71.

CDC values for a given indicator is performed between values obtained at identical plasma flows. Table III summarizes literature values of CDC for sucrose and $^{51}\text{Cr EDTA}$ which for all practical purposes have same diffusion and permeability characteristics. Good agreement with the results of the present series is seen, when account is taken for the plasma flows. In "hyperemic" skeletal muscle in man $\text{CDC}(^{57}\text{Co-B12})$ was $1.7 \text{ ml}/100 \text{ g min}$ and $\text{CDC}(^{51}\text{Cr EDTA})$ was $3.4 \text{ ml}/100 \text{ g min}$ (Lassen and Trap-Jensen 1968a). These values correspond to the results of the present series. At a plasma flow of $7 \text{ ml}/100 \text{ g min}$ $\text{CDC}(^{57}\text{Co-B12})$ can be calculated to $1.9 \text{ ml}/100 \text{ g min}$ from the equation $y = 1.43 \cdot 0.071x$. At $S = 70 \text{ cm/g}$ and $f(\text{pl}) = 7 \text{ ml}/100 \text{ g min}$, $P_d(^{57}\text{Co-B12})$ becomes $0.45 \cdot 10^{-6} \text{ cm}^2/\text{sec}$. The permeability of the capillary wall is $0.74 \cdot 10^{-6} \text{ cm}^2/\text{sec}$. The permeability of the

TABLE III. Capillary diffusion capacity, CDC, and permeability coefficient, P_d , for sucrose and ^{51}Cr EDTA in skeletal muscle as measured by methods based on indicator diffusion.

Indicator	CDC ml/100 g min mean (range)	P_d 10^4 cm/s mean	Plasma flow fl(pl) ml/100 g min mean (range)	Method	Species & Tissue	Reference
Sucrose	3.1	0.74	~ 7.2	ID	Dog hindlimb	Crone 1963
^{51}Cr EDTA	$\begin{Bmatrix} 4.8 (3.5-5.9) \\ 4.4 (3.5-6.1) \end{Bmatrix}$	$\begin{Bmatrix} 1.14 \\ 1.04 \end{Bmatrix}$	14.7 (11.6-23.3)	$\begin{Bmatrix} \text{ID} \\ \text{SI} \end{Bmatrix}$	Cat gastroc muscle	Sejrsen 1970 and pers. comm.
Sucrose	2.6 (2.3-4.7)	0.86	14.1 (6-26)	ID	Horse forelimb	Trap-Jensen and Lassen 1970
^{51}Cr EDTA	3.5 (2.4-4.9)	0.83	12.9 (10-31)	TC	Horse sub. m.	Trap-Jensen et al 1970
^{51}Cr EDTA	3.2 (1.5-4.5)	0.77	~ 14 (4.4-23.4)	ID	Horse forelimb	Trap-Jensen and Lassen 1971
^{51}Cr EDTA	5.5 (2.0-10.0)	$\begin{Bmatrix} 0.34 \text{ at fl(pl) } \sim 7 \\ 1.02 \text{ at fl(pl) } 15 \\ 1.30 \text{ at fl(pl) } \sim 23.2 \end{Bmatrix}$	23 (4.0-52.4)	SI	Cat gastroc muscle	Paske 1977 (present study)

Abbreviations: ID: indicator diffusion method, SI: single injection, external counting method, TC: tissue clearance method.

Note: All P_d values are calculated as: capillary surface area, S , of $70 \text{ cm}^2/\text{g}$. From the arguments presented in the discussion, S must be considered as accurate with increasing plasma flow. Normalizing with respect to this phenomenon, P_d becomes $\sim 1.5 \cdot 10^{-4} \text{ cm/s}$ for the present series. A capillary surface area of $35 \text{ cm}^2/\text{g}$ is probably better estimate at plasma flow of $7 \text{ ml}/100 \text{ g min}$.

EDTA and $^{51}\text{Co-B12}$ are in agreement with results obtained in other tissues with capillaries of continuous type (Paske 1977 a). The results support the hypothesis suggested previously that capillaries of continuous type exhibit same permeation characteristics regardless of the tissue in which they are located (Paske 1976).

III. *Capillary pore size* The ratio between the free diffusion coefficients in water at 37°C is $D(^{51}\text{Cr EDTA})/D(^{51}\text{Co-B12}) = 0.700 \cdot 10^{-4} (\text{cm}^2/\text{s})/0.390 \cdot 10^{-4} (\text{cm}^2/\text{s}) = 1.79$ (Paske 1977 a). In the present series $\text{CDC}(^{51}\text{Cr EDTA})/\text{CDC}(^{51}\text{Co-B12}) \sim 1.79$ which implies that the diffusion of these indicators across the capillary membrane of skeletal muscle takes place with rate constants proportional to the free diffusion coefficients of the two indicators. The data can therefore obviously not be applied for calculation of an equivalent pore radius. The concept of a circular and cylindrical water-filled pore of 30 \AA radius or—alternatively—sin width of 37 \AA was introduced by Pappenheimer, Renkin and Borrero (1951). Applying the biogravimetric osmotic transient method to the cat hindlimb preparation it was found that the permeability coefficients for series of indicators decreased more than could be anticipated from the decrease in free diffusion coefficients. From analysis employing the theory of restricted diffusion (Faxén 1922, Ferry 1936) the 30 \AA pore radius estimate was obtained. This estimate was questioned by Crone (1963 b) who applied the "indicator diffusion method" (Crone 1963 a) to the dog hindlimb preparation. This technique is not complicated by osmotic reflection coefficient corrections and the finding of absence of restricted diffusion to insulin as compared to sucrose leads to a considerably larger pore radius estimate.

SKELETAL MUSCLE
 ^{57}Co CYANOCOBALAMIN

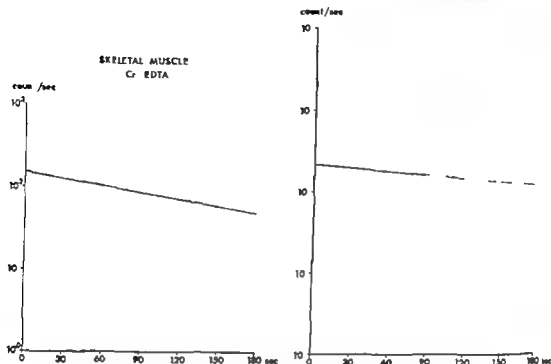


Fig. 5 Fig. 5a presents the computer printout of the recorded count values for ^{54}Cr EDTA corrected for background activity (9 cps) plotted as a function of time on a semilogarithmic diagram (dots) (Expt. No. 9). At time zero the bolus injection was given. A maximum count value of 617 cps was reached at 2nd s (peak time). The part of the curve recorded in the time interval 60 s–180 s obeyed the equation $\text{cps}(t) = 151 \exp(-0.0063 t)$ where t is time in s. The standard deviation on the slope, $\text{SD}(k)$, was 0.0003 s. At peak time (2nd s) value of 150 was obtained. Capillary extraction, $E(^{54}\text{Cr}$ EDTA), was $150/617 = 0.243$. Plasma flow as measured directly was 20.9 ml/100 g min and $\text{CDC}(^{54}\text{Cr}$ EDTA) = $-20.9 \cdot 0.89 \ln(1 - 0.243)$ (ml/100 g min) = 5.2 ml/100 g min. As the mean transit time of ^{54}Cr EDTA in plasma was 0.108 min (crosses denote the vascular transit) the plasma flow could be calculated from $f(p) = (1/0.108) \cdot (1 - 0.266) = 0.03 \cdot 100$ (ml/100 g min) = 3.0 ml/100 g min.

The simultaneously recorded ^{57}Co -B12 tracing is given in Fig. 5b, where count values corrected for cross-talk and background activity are plotted as a function of time on a semilogarithmic diagram. Cross-talk correction factor from ^{54}Cr to ^{57}Co was 0.248. Background value for ^{57}Co -B12 was 22 cps. Maximum count value, 1310 cps, was reached at 1 s. The extrapolation slope was given by $\text{cps}(t) = 215 \exp(-0.0030 t)$ with $\text{SD}(k) = 0.0003 \text{ s}^{-1}$. $E(^{57}\text{Co}$ -B12) was $215/1310 = 0.164$ giv. g $\text{CDC}(^{57}\text{Co}$ -B12) = 3.0 ml/100 g min. As determined from kinetic analysis of the ^{57}Co -B12 curve plasma flow is 18.5 ml/100 g min. The ratio $\text{CDC}(^{54}\text{Cr}$ EDTA)/ $\text{CDC}(^{57}\text{Co}$ -B12) was 1.73.

CDC values for a given indicator is performed between values obtained at identical plasma flows. Table III summarizes literature values of CDC for sucrose and ^{54}Cr EDTA which for all practical purposes have same diffusion and permeability characteristics. Good agreement with the results of the present series is seen, when account is taken for the plasma flows. In "hyperemic" skeletal muscle in man $\text{CDC}(^{57}\text{Co}$ -B12) was 1.7 ml/100 g min and $\text{CDC}(^{54}\text{Cr}$ EDTA) was 3.4 ml/100 g min (Lassen and Trap-Jensen 1968 a). These values correspond to the results of the present series. At a plasma flow of 7 ml/100 g min $\text{CDC}(^{57}\text{Co}$ -B12) can be calculated to 1.9 ml/100 g min from the equation $y = 1.43 \cdot 0.071x$. At $S = 70 \text{ cm}^2/\text{g}$ and $f(p) = 7 \text{ ml/100 g min}$, $P_a(^{57}\text{Co}$ -B12) becomes $0.45 \cdot 10^{-3} \text{ cm/s}$ and $P_a(^{54}\text{Cr}$ EDTA) correspondingly $0.74 \cdot 10^{-3} \text{ cm/s}$. The permeabilities of ^{54}Cr

TABLE III. Capillary diffusion capacity CDC, and permeability coefficient, P_e , for sucrose and ^{54}Cr -EDTA in skeletal muscle as measured by methods based on indicator diffusion.

Indicator	CDC ml/100 g min mean (range)	P_e 10^6 cm/s mean	Plasma flow l(pl) ml/100 g min mean (range)	Method	Species & Tissue	Reference
Sucrose	3.1	0.74	~ 7.2	ID	Dog hindlimb	Croce 1963
^{54}Cr -EDTA	$\left\{ \begin{array}{l} 4.8 (3.5-8) \\ 4.4 (3.5-6.1) \end{array} \right\}$	$\left\{ \begin{array}{l} 1.14 \\ 1.04 \end{array} \right\}$	14.7 (11.6-23.3)	$\left\{ \begin{array}{l} \text{ID} \\ \text{SI} \end{array} \right\}$	Cat gastroc muscles	Sejrsen 1970 and pers. comm.
Sucrose/ ^{54}Cr -EDTA	3.6 (2.2-4.7)	0.84	14.1 (6-25)	ID	Human forearm	Trap-Jensen and Lassen 1970
^{54}Cr -EDTA	3.3 (2.2-4.9)	0.83	18.9 (10-31)	TC	Human ant. t b. m.	Trap-Jensen et al. 1970
^{54}Cr -EDTA	3.2 (1.3-4.3)	0.77	~ 14 (4.4-34)	ID	Human forearm	Trap-Jensen and Lassen 1971
^{54}Cr -EDTA	5.5 (2.0-10.0)	$\left\{ \begin{array}{l} 0.74 \text{ at } R(p) = 7 \\ 1.02 \text{ at } R(p) = 15 \\ 1.30 \text{ at } R(p) = 23 \end{array} \right\}$	23.2 (4.0-52.4)	SI	Cat gastroc muscles	Paske 1977 (present study)

Abbreviations: ID: indicator diffusion method, SI: single injection, external counting method, TC: tissue clearance method.

Note: All P_e values are calculated at capillary surface area, S , of $30 \text{ cm}^2/\text{g}$. From the regressions presented in the discussion, S must be considered to increase with increasing plasma flow. Normalizing with respect to this phenomenon, P_e becomes $\sim 1.5 \cdot 10^{-6} \text{ cm/s}$ for the present series. A capillary surface area of $33 \text{ cm}^2/\text{g}$ probably better estimates plasma flow of 7 ml/100 g min.

EDTA and $^{54}\text{Co-Bi2}$ are in agreement with results obtained in other tissues with capillaries of continuous type (Paske 1977). The results support the hypothesis suggested previously that capillaries of continuous type exhibit same permeation characteristics regardless of the tissue in which they are located (Paske 1976).

B. Capillary pore size The ratio between the free diffusion coefficients in water at 37°C is $D(^{54}\text{Cr-EDTA})/D(\text{Co-Bi}) = 0.700 \cdot 10^{-6} (\text{cm}^2/\text{s})/0.390 \cdot 10^{-6} (\text{cm}^2/\text{s}) = 1.79$ (Paske 1977). In the present series $\text{CDC}(^{54}\text{Cr-EDTA})/\text{CDC}(\text{Co-Bi2}) = 1.79$ which implies that the diffusion of these indicators across the capillary membrane of skeletal muscle takes place with rate constants proportional to the free diffusion coefficients of the two indicators. The data can therefore obviously not be applied for calculation of an equivalent pore radius. The concept of circular and cylindrical water-filled pore of 30 \AA radius or—alternatively—slit width of 37 \AA was introduced by Pappenheimer, Renkin and Borrero (1951). Applying the microgravimetric osmotic transient method to the cat hindlimb preparation it was found that the permeability coefficients for a series of indicators decreased more than could be anticipated from the decrease in free diffusion coefficients. From analysis employing the theory of restricted diffusion (Faxén 1922, Ferry 1936) the 30 \AA pore radius estimate was obtained. This estimate was questioned by Croce (1963 b) who applied the indicator diffusion method (Croce 1963 a) to the dog hindlimb preparation. This technique is not complicated by osmotic reflection coefficient corrections and the finding of absence of restricted diffusion to inulin as compared to sucrose leads to a considerably larger pore radius estimate.

By an alternative indicator diffusion technique this finding has recently been confirmed (Paaske and Sejrsen 1977). From studies based on filtration expts. pore radius estimates from 31 to 45 Å have been obtained (Grotte 1956, Renkin and Pappenheimer 1957, Landis and Pappenheimer 1963, Diana, Long and Yao 1972) whereas studies of diffusion permeability (Crone 1963 b, Lassen and Trap-Jensen 1968 a, Paaske and Sejrsen 1977, present study) showed absence of restricted diffusion for molecules in the molecular weight range 340–5 500. Yet, Trap-Jensen and Lassen (1970, 1971) found massive restriction for molecules in the same range by the "indicator diffusion" method applied to the human forearm (slit width estimate 40 Å).

The findings of the present study are incompatible with the belief that transcapillary exchange of hydrophilic solutes in skeletal muscle takes place through the Karnovsky 40 Å interendothelial slit which has been speculated to be morphological equivalent for the Pappenheimer pore (Karnovsky 1967, 1968). However, in a recent study by Simionescu, Simionescu and Palade (1975) a channel system of fused intraendothelial vesicles was demonstrated by electron micrographs in the continuous capillary of skeletal muscle. Although the significance of this system is not established as yet it appears that transendothelial passage of lipid-insoluble molecules through such a system is compatible with permeability data obtained in a number of physiological studies (Kruhoffer 1946, Crone 1963 b, Lassen and Trap-Jensen 1968 a, Alvarez and Yudilevich 1969, Boyd *et al.* 1969, Normand *et al.* 1971, Bassingthwaite, Yipintsoi and Grabowski 1975, Paaske 1977 a and b, Paaske and Sejrsen 1977). Considering the compiled data from studies based on indicator diffusion it would seem reasonable to believe that the equivalent pore radius is at least 90 Å. Recent data from lung (Perl, Chowdhury and Chinnard 1975) and frog mesentery (Curry, Mason and Michel 1976) have questioned the belief that the magnitude of the osmotic reflection coefficient increases with increasing molecular size of the test indicator for these continuous capillary membranes. Considering the experimental errors in the studies mentioned above these findings might be accounted for by assuming a larger pore than is conventionally considered based on measurements of filtration. The lower pore radius estimate of filtration studies might therefore be defective due to incomplete experimental knowledge of the reflection coefficients and the possibility that an alternative pathway for water filtration exists in addition to the pathway for the hydrophilic solutes. These possibilities might contribute to the "Pappenheimer pore puzzle" as Perl's (1971) solution to this problem was based on a 40 Å slit width and becomes defective with a pore radius so large as it does not exhibit significant restricted diffusion to Co-B₁₂ compared with ⁵¹Cr-EDTA. The present data also exclude the necessity for a large pore system and vesicular transport of these hydrophilic molecules is excluded since their transport takes place with rate constants proportional to the free diffusion coefficients.

Assuming a pore radius of 90 Å the pore area, S_p , can be calculated as $S_p = CDC \Delta x_d / D$ (cm²/g) where Δx_d is diffusion distance across the endothelium. As the "patent channel" system seems to be located perpendicularly to the basement membrane we consider $\Delta x_d \sim 1\,000$ Å. At CDC (⁵¹Cr-EDTA) = 3.7 ml/100 g min $S_p = ((3.7/100 \cdot 60) \cdot 1 \cdot 10^{-7}) / (0.7 \cdot 10^{-4} - 8.8 \cdot 10^{-4})$ cm²/g. At $S = 70$ cm²/g we obtain $S_p/S = 1.26 \cdot 10^{-4}$ i.e. the pore area available for transendothelial diffusion is $\sim 1/100\,000$ of the total capillary surface area.

The number of pores per g tissue which at any time must be open is $S_p/\pi r^2 = 8.8 \cdot 10^{-4} / 2.54 \cdot 10^{-11} = 3.5 \cdot 10^6$. Approximately 175 capillaries are open in 1 mm of skeletal muscular tissue at a plasma flow corresponding to the chosen CDC (^{51}Cr EDTA) (Landis and Pappenheimer 1963). This leads to $3.5 \cdot 10^6 / 175 = 2 \cdot 10^4$ open pores per capillary. At a capillary length of 1200 μm we get an estimate of ~ 1.2 open pores per μm capillary length. This number could very well explain the fact that channels of fused vesicles are rarely seen on electron micrographs.

C. Recruitment The present study indicates (Table I, Figs. 2 and 4) that CDC for the two indicators increases 3–3.5 fold from resting muscle ($f(p) = 4 \text{ ml}/100 \text{ g min}$) to heavily exercising muscle ($f(p) = 52 \text{ ml}/100 \text{ g min}$). Three possible explanations for this phenomenon can be considered: 1) constant capillary surface area with increased pore density times pore cross sectional area, 2) increased capillary surface area with constant pore density times pore cross sectional area, 3) increased capillary surface area with increased pore density times pore cross sectional area. As permeability coefficient and surface area are inseparable in their product no conclusive statement as regarding the validity of either explanation can be obtained from the present data. From studies on the capillary filtration coefficient, CFC, of the Starling formulation of transcapillary fluid movement, it is known that CFC increases 3–4 fold with transition from resting to exercising state in hind part in cats (Cobbold *et al.* 1963) and in cat calf muscles (Kjellmer 1964). As argued by Kjellmer (1964) CFC should increase in proportion to the 4th power of the pore radius (Poisuille's law) while CDC for hydrophilic solutes would increase with the 2nd power (Fick's law), if an increase in pore size is responsible. At a CDC increase of factor 3 CFC should consequently increase 9 fold. However if capillary surface area is responsible CFC should increase 3 fold. Considering the CFC data and this argumentation it would seem reasonable to assume that the CDC and CFC increases should be attributed to an increased capillary surface area rather than an increase in pore size. From histological studies it is known that the number of open capillaries per unit cross sectional area of skeletal muscle varies (Landis and Pappenheimer 1963, Hasselmersen 1968). A part of the blood to tissue exchange area might be positioned in venules. For this reason, it is doubtful whether CDC and CFC data can be correlated rigorously to capillary counts.

By the criticized (Cros 1970) continuous infusion method Renkin, Hudlická and Sheehan (1966) obtained results indicating that CDC for ^{86}Rb and ^{42}K increased 2.5 fold in the flow range from 7.9 to 30.2 $\text{ml}/100 \text{ g min}$ in canine gracilis muscle. An increase of CDC for hydrophilic indicators with increasing plasma flow has not been demonstrated in other studies in skeletal muscle. This is probably due to the fact that all other comparable studies (Table III) have operated at plasma flows of 31 $\text{ml}/100 \text{ g min}$ or lower. In other tissues CDC for various indicators has recently been shown to be linearly related to plasma flow in myocardium (L. Abbate *et al.* 1976), lung (Basset *et al.* 1974), and submandibular gland (Mann, Smajic and Yudilevich 1976).

Assuming as above that CDC is linearly related to capillary surface area, S , and assuming capillary radius 3.2 μm , the ordinate axis on Fig. 4 can be considered to provide an estimate of surface area of perfused capillaries in cm^2/g by dividing the ordinate axis values by two. The consequences of these considerations are that capillary surface area of $\sim 35 \text{ cm}^2/\text{g}$

would probably be a more correct estimate for plasma flows ~ 7 ml/100 g min leading to P_e values of $\sim 1.5 \cdot 10^{-7}$ cm/s and $\sim 0.9 \cdot 10^{-8}$ cm/s for ^{51}Cr EDTA and ^{57}Co -B12, respectively. Further, it can be calculated that a capillary volume of ~ 12 per cent of total vascular volume is present in resting condition whereas in maximum exercise ~ 50 per cent of the vascular volume will be located in exchange vessels. These considerations are in agreement with Landis and Pappenheimer (1963). This has not been taken into account with the kinetic calculation of plasma flow where a vascular volume of 0.03 ml/g has been used in all instances as a reasonable estimate. This was based on the fact that the experiments did not provide evidence for an increase of total intravascular volume. The intermittent mechanical compression of the vessels during exercise might influence an eventual redistribution of blood volume within the vascular system of the muscle.

The mean transit time for the indicators in the capillaries can be calculated from $t(\text{cap}) = V(\text{cap})/f(\text{pl})$, where $V(\text{cap})$ is intracapillary plasma volume. Assuming $V(\text{cap})$ at rest to be 12 per cent of the total intravascular volume, the mean transit time at resting perfusion (4 ml/100 g min) becomes $t(\text{cap-rest}) = 0.03 \cdot 0.12 \cdot (1 - H_{ct}) \cdot 100 \cdot 60/4 \sim 4$ s. During maximum exercise ($f(\text{pl}) = 52$ ml/100 g min) $V(\text{cap})$ is increased with the factor 3-3.5. Consequently $t(\text{cap-exercise}) = 0.03 \cdot 0.50 \cdot (1 - H_{ct}) \cdot 100 \cdot 60/52 \sim 1.2$ s. The linear velocity of the blood will increase with a factor of $t(\text{cap-rest})/t(\text{cap-exercise}) = 4/1.2 \sim 3.5$. When the number of functioning capillaries is increased with a factor 4 from rest to maximum exercise this will reduce the intercapillary distances with a factor 2, resulting in a reduction in diffusion time with a factor 4. This reduction in diffusion time will compensate for the 3.5 fold increase in linear velocity.

D. Back-diffusion. Back-diffusion of indicator is defined as early reentry of indicator from interstitial fluid to blood while the indicator bolus is still in exchange vessels. Capillary permeability will consequently be underestimated. In the present series significant positive correlation could not be demonstrated between $\text{CDC}(\text{ }^{51}\text{Cr EDTA})/\text{CDC}(\text{ }^{57}\text{Co-B12})$ and plasma flow / *i.e.* there is no reason to believe that back-diffusion occurs to a significant extent for the employed indicators within the observed plasma flow range of 4-52 ml/100 g min (Fig. 3), and no experimental validation was found for the assumption (Schafer and Johnson 1964) that extraction of ^{51}Cr EDTA (and ^{57}Co -B12) should be blood flow limited. This finding is of importance for interpretation of measured $\text{CDC}(\text{ }^{51}\text{Cr EDTA})$ and $\text{CDC}(\text{ }^{57}\text{Co-B12})$ values obtained in the continuous capillary bed of cutaneous and adipose tissues in rabbits in which the physiological plasma flow range is from 4 to 10 ml/100 g min (Paaske and Nielsen 1976, Paaske 1976, 1977 a and b). On this basis it is unlikely that these results are influenced by back-diffusion. In adipose tissue which has about half the capillary surface area of skeletal muscle it should be possible to perform CDC determinations with these indicators without back-diffusion even at plasma flows of some 2 ml/100 g min. The position of individual patent channels is probably a stochastic variable. If the dynamic processes of formation and degradation of vesicles are sufficiently fast it would seem improbable that an individual molecule will pass from interstitium to blood via the same pathway through which it entered the interstitium. The resulting increase in interstitial diffusion distance will reduce the probability for back-diffusion while the bolus is still located within exchange vessels.

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The Effect of Indomethacin on Renal Blood Flow Distribution during Hemorrhagic Hypotension in Dog

By

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Abstract

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The effect of indomethacin on hemorrhagic hypotension (HH) on total renal blood flow (RBF) and cortical flow distribution was studied in 9 pentobarbital anesthetized dogs. Local blood flow was measured as hydrogen clearance by 6 platinum electrodes in outer and inner cortex. RBF as recorded by electromagnetic flowmeter. Injection of indomethacin (3-5 mg/kg b.wt.) to inhibit prostaglandin synthesis, reduced renal blood flow only a few per cent in control period. After the pretreatment with indomethacin, bleeding to mean arterial pressure 30 mmHg decreased RBF abruptly to 25% of control flow. Thus renal vasoconstriction is observed both in early and late periods of bleeding in contrast to the arterial vasodilation observed during the first half an hour of HH, when prostaglandin formation is not blocked. Local blood flow in outer cortex decreased proportionally to flow in inner cortex during bleeding in both the indomethacin treated as in the untreated group, indicating an equal vasodilatory effect of prostaglandins in all parts of renal cortex during early periods of HH.

Several studies of renal hemodynamics in hemorrhagic hypotension (HH) have shown an initial reduction of the renal vascular resistance, followed by a marked increase of resistance in both cortex and medulla after 1/2-1 h (Jirka *et al.* 1961, Aukland and Wolgast 1968, Aukland *et al.* 1973, Kirkebo and Tyseboth 1974). While Aukland and Wolgast (1968) proposed that this resistance fall might be a kind of autoregulatory response to the decreased blood pressure, it still remains to describe the mechanisms involved in such a response. Prostaglandin E (PGE) is a possible mediator as it was recently shown that the concentration of PGE in arterial blood is markedly increased during HH (Flynn *et al.* 1975, Johnston and Selkurt 1976). Since a number of studies have located the renal PGE synthesis to the outer medulla (Leo *et al.* 1967), vasodilation caused by endogenous PGE would be expected to be limited to outer medulla and/or the deepest part of inner cortex. This would be compatible with findings of especially low flow in inner cortex after blockade of PGE synthesis with indomethacin during HH (Data *et al.* 1976), but does not fit with our findings of an unchanged cortical flow distribution during HH (Aukland *et al.* 1973, Kirkebo and Tyse-

both 1974). It is possible, however, that without any release of PGE, the vasoconstriction would have been even more marked in the inner cortex.

In view of these considerations we wanted to find out whether indomethacin blockade of PGE synthesis would alter the pattern of resistance change during HH—and if so, whether the effect would be limited to certain parts of the cortex.

All results in the present study are compared to results from a series of expts. made with identical procedure, but without use of any blockers (Aukland *et al.* 1973).

Methods

Experiments were made on 9 mongrel dogs of both sexes, weighing 16–23.5 kg. Anesthesia was induced by pentobarbital 25 mg/kg b.wt. and additional doses given when needed, usually about 3 mg/kg b. The dogs had free access to water but no food was given for 16 h prior to anesthesia.

Polyethylene catheters were placed in femoral artery and vein for bleeding and infusions respectively. A catheter was placed in brachial artery for pressure recording.

The kidney was exposed retroperitoneally through flank incision and the renal artery gently dissected free. A polyvinyl catheter was sown into the renal artery (Herd and Barger 1964) for upstream injection of H₂ saturated saline heated to 37°C. An electro-magnetic flow probe was placed on the renal artery for total renal blood flow measurement (Nycotron). The flowmeter was calibrated directly in femoral artery.

L-shaped platinum electrodes were inserted to different depths of the cortex so that 3 had the sensor electrode tip in outer half of the cortex, and 3 in the inner half. The electrodes were 0.2 mm in diameter and made of 90% platinum and 10% iridium coated with an enamel lacquer. The sensitive electrode tip was made by stripping off the lacquer from 0.5 mm of the electrode tip.

H₂ saturated saline was injected until constant current was measured polarographically on a Rikadenki recorder (Kogro B64) at constant polarization potential of +0.2 V vs. an Ag/AgCl electrode—then the injection was suddenly stopped and the washout curve recorded. The flow in ml/min g was determined from the formula $K = \ln 0.5/T_{1/2}$ where $T_{1/2}$ in min is the half-life of the H₂ current (Aukland *et al.* 1964). All washout curves were monoexponential down to 10% of initial saturation when plotted on a semi-logarithmic paper and $T_{1/2}$ was easily determined.

Control measurements were made the last 20 min before bleeding was started. Indomethacin was given i.v. in doses of 3–5 mg/kg b.wt. about 10–15 min before bleeding started, and 3 measurements of RBF and local flow were made after indomethacin was given.

Bleeding procedure

The dog was bled to a mean arterial pressure (\bar{AP}) of 50 mmHg, 5–20 min by free flow through the catheter in the femoral artery into a reservoir placed 68 cm above heart level. Heparin (3000 I.U.) was given into the reservoir. H₂ washout curves were made with few minutes intervals in the first half hour of bleeding, later with intervals of about 20 min, until retransfusion started 2 h after the initiation of bleeding. Reinfusion of the heparinized reservoir blood induced profuse bleeding in 6 dogs, making measurements after retransfusion inappropriate.

At the end of the expt. the kidney was excised and the position of each electrode carefully examined. All electrodes were located in the right cortical half and no electrodes were excluded. The drained kidney weight was used for calculation of RBF (ml/min g).

The present measurements are compared to results from a previously published study (7 expts.) (Aukland *et al.* 1973) where the surgical and bleeding procedure as well as technical equipment were identical.

Using paired t-tests for statistical evaluation, differences were considered significant if $p < 0.05$.

Results

Control measurements

The average mean arterial pressure (\bar{AP}) was 124 mmHg, with a range of 110 to 140 mmHg. At the same time the renal blood flow (RBF) averaged 3.50 (\pm S.D. 0.79) ml/min g. At the same

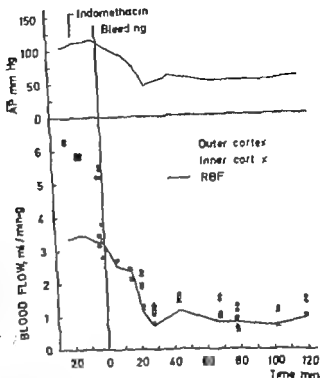


Fig. 1 Effect of indomethacin and bleeding on mean arterial pressure (\overline{AP}), total (RBF) and local blood flow at 6 separate electrode sites in outer and inner cortex of one dog

the average outer cortical blood flow (OCF) and inner cortical blood flow (ICF) were 3.57 (\pm S.D. 0.88) and 3.43 (\pm S.D. 0.60) ml/min/g respectively. This gives a ratio OCF/ICF of 1.04 which is not significantly different from unity.

After one I.v. injection of indomethacin $3-5$ mg/kg b.wt. \overline{AP} increased only 3.1% from control level whereas RBF decreased 10.2% ($p < 0.05$). Total renal vascular resistance increased 14.8% ($p < 0.02$). Local blood flow and its distribution changed little as OCF and ICF decreased 6.1% and 6.0% respectively (OCF/ICF ratio 1.04). These data indicate a slight rise in vascular resistance in all cortical layers in the kidney. The absolute flow values agreed well with control values in a previous study (Aukland *et al.* 1973) finding that RBF, OCF and ICF were 5.30 , 3.47 (\pm S.D. 0.83) and 3.61 (\pm S.D. 0.86) ml/min/g respectively. Average \overline{AP} was 118 mmHg.

Hemorrhagic hypotension

All dogs were bled $900-1000$ ml to reach an \overline{AP} of 50 mmHg within 20 min, and this blood pressure level was maintained for about 2 h before beginning retransfusion of shed blood. U_e clearances were measured every $5-10$ min in the first half hour of bleeding, later every 20 min.

RBF fell rapidly during bleeding, even faster than the fall in \overline{AP} . Local blood flow at all electrode sites in outer and inner cortex fell simultaneously and to the same degree as the RBF as shown for a single experiment in Fig. 1.

botn 1974). It is possible, however, that without any release of PGE, the vasoconstriction would have been even more marked in the inner cortex.

In view of these considerations we wanted to find out whether indomethacin blockade of PGE synthesis would alter the pattern of resistance change during HH—and if so, whether the effect would be limited to certain parts of the cortex.

All results in the present study are compared to results from a series of expts. made with identical procedure, but without use of any blockers (Aukland *et al.* 1973).

Methods

Experiments were made on 9 mongrel dogs of both sexes, weighing 18–23.5 kg. Anaesthesia was induced by pentobarbital 25 mg/kg b.wt. and additional doses given when needed, usually about 3 mg/kg. The dogs had free access to water but no food was given for 16 h prior to anaesthesia.

Polyethylene catheters were placed in a femoral artery and vein for bleeding and infusions respectively. A catheter was placed in a brachial artery for pressure recording.

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Bleeding procedure

The dog was bled to a mean arterial pressure (\bar{AP}) of 50 mmHg in 5–70 min by free flow through the catheter in the femoral artery into a reservoir placed 60 cm above heart level. Heparin (5000 IU) was given into the reservoir. H_2 washout curves were made with few min intervals in the first half hour of bleeding, later with intervals of about 70 min. If retransfusion started 2 h after the initiation of bleeding. Reinfusion of the heparinized reservoir blood induced profuse bleeding in all dogs, making measurements after retransfusion inappropriate.

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The present measurements are compared to results in previously published study (7 expts.) (Aukland *et al.* 1973) where the surgical and bleeding procedure as well as technical equipment were identical.

Using paired *t* tests for statistical evaluation, differences were considered significant at $p < 0.05$.

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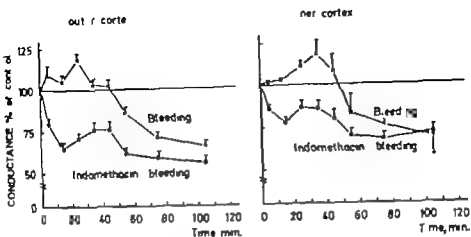


Fig 3 Relative vascular conductance (\pm S.E.) in outer and inner renal cortex during bleeding (7) (crosses) and during bleeding after pretreatment with indomethacin (8) (open circles)

RBF respectively after injection of indomethacin in pentobarbital anesthetized dogs. In conscious dogs, however Swain *et al.* (1975) found only 12% decrease in renal blood flow while renal resistance increased 15% after indomethacin treatment in accordance with our results on anesthetized dogs, suggesting that the effect of the anesthetization and the handling of the kidney is minimal in our study.

Total renal blood flow during bleeding. The renal response to hemorrhage has classically been described as a vasoconstriction (Trueta *et al.* 1947) whilst more recently published studies have shown that even a vasodilation may occur especially in the first period of HH (Jarka *et al.* 1961, Anklund and Wolgast 1968, Kirkebo and Tysseboen 1974). In our previous study (Anklund *et al.* 1973) using identical experimental procedure as in the present experiments, a fall in vascular resistance was observed during the first 30 min of bleeding, followed by a steady increase in resistance, probably caused by an initial autoregulatory response which gradually is overpowered by increasing vasoconstrictor activity.

According to several investigators (Anderson *et al.* 1975, Venuto *et al.* 1975), however indomethacin does not influence renal autoregulation. Nevertheless, renal autoregulatory response has been studied by adjustable clamp on aorta at unchanged blood volume, and *a vasopressor activity is much less in such studies than in studies where the blood volume markedly reduced by bleeding.* Pretreatment with indomethacin also is reported to potentiate the renal vasoconstrictor response of renal nerve stimulation, infusion of noradrenalin: angiotensin (Needleman *et al.* 1976).

Our previous results are contrary to the findings in the present study where bleeding to 70 mmHg after treatment with indomethacin resulted in an immediate increase in renal vascular resistance, probably explained by an augmented vasopressor activity in this group.

Since the prominent effect of indomethacin is to block PGE synthesis, it is indicated that the observed vasodilation in the first phase of HH was induced by PGE. This is supported by the finding of increased concentration of PGE in arterial blood during HH reported by

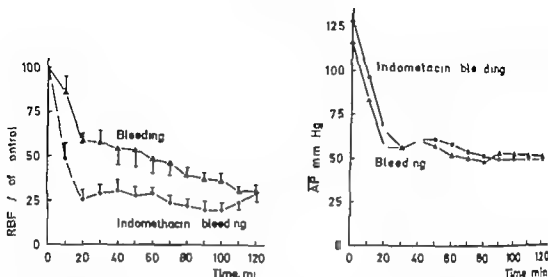


Fig. 2. Average total renal blood flow (RBF) (\pm S.E.) and arterial pressure (AP) during bleeding ($n = 7$) (triangles) and during bleeding after pretreatment with indomethacin ($n = 9$) (circles).

Generally in the indomethacin treated dogs RBF in the early bleeding phase decreased abruptly to 25% of initial flow and thereafter was practically unchanged for the rest of the expt. (Fig. 2). In the untreated group however RBF fell only to 60% of control during the first 20 min, but thereafter showed a gradual decrease of flow for 1.5 h. In both groups average AP fell to the same degree during the first 20 min and ended at the same pressure within 30 min, thus renal vascular resistance was markedly higher in the indomethacin treated group. After about 2 h of hypotension relative RBF in the two groups were almost identical and about 30% of initial control. Similar differences in flow reduction in the early phase of bleeding, of indomethacin treated and untreated dogs, were found also by the local blood flow measurements in outer and inner cortex. The volume bled from the indomethacin treated dogs was in order of 0.7 l, which was distinctly lower than the untreated group (1.2 l) suggesting an improved compensation of the blood volume loss in the last group.

The vascular conductance of outer and inner cortex (OCF/AP and ICF/AP) of the indomethacin treated dogs showed an immediate great reduction during the first 15 min of bleeding (Fig. 3). A short lasting increase in conductance occurred as the bleeding velocity was decreased at AP about 50 mmHg (in 1 dog ICF/AP for some minutes rose even above initial control). After about 1 h average vascular conductance in outer and inner cortex was reduced to a level of approximately 60–70% of prebleeding value.

The untreated dogs showed opposite conductance changes for the first period of HH as the conductance for the whole kidney and different cortical layers increased in all but 1 animal. The average conductance was above control for 40–50 min before the conductance decreased to the same level as in the indomethacin treated dogs.

Discussion

Intravenous injection of indomethacin under control conditions changed neither total renal blood flow nor cortical flow distribution by more than a few per cent. Kirichenbaum *et al* (1974) and Swain *et al* (1975) on the other hand reported 45 and 25% decrease of

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several authors (Flynn, Appert and Howard 1975 and Johnston and Selkurt 1976). As vascular resistance and renal blood flow after 2 h bleeding was similar in the present 2 series of expts. the vasodilation caused by PGE release must be temporary or its effect suppressed by increased vasoconstrictor influence also in the untreated group.

Renal blood flow distribution Carriere *et al* (1966), Truninger *et al* (1966) and other using ^{86}Kr and ^{133}Xe washout technique, reported a reduced blood flow in outer cortex during HH while blood flow in juxtamedullary cortex and outer medulla was well preserved. These results have been supported by Slotkoff *et al* (1971) and Rector *et al* (1977) showing an increased fraction of microspheres to inner cortical nephrons in hypotensive state. Data *et al* (1976) demonstrated a pronounced reduction in blood flow to inner cortex as well during HH after pretreatment with indomethacin. According to Lee *et al* (1967) renal PGE production is limited to outer medulla and Data *et al* (1976) therefore suggested that renal PGE was responsible for the selective vasodilation in inner cortex during bleeding.

On the other hand, Aukland *et al* (1973) and Kirkebo and Tysebotn (1974) found no evidence for a redistribution of cortical blood flow during HH and Aukland and Wolgast (1968) measured a proportional reduction in total renal and outer medullary blood flow. Furthermore after pretreatment with indomethacin in the present study local blood flow in outer and inner cortex showed a similar decrease during bleeding. A vasoconstrictor modulation or a vasodilatory effect of the increased arterial PGE content during bleeding, whether this is due to increased PGE synthesis outside the kidney or increased passage through the lung, probably will affect outer and inner cortical vasculature to the same degree, thus compatible with the present results.

Johnston and Selkurt (1976) did not find increased renal release of PGE 1-3 h after started bleeding, but they performed no measurements in the first hour of bleeding, so the renal release may well be higher this period. Jakachik *et al* (1974) reported peak arterial PGE concentration after half an hour hemorrhage, when the discrepancy between the two groups are greatest in our study.

If equal changes of blood flow in both cortical zones anyhow depends on endogenous renal release of PGE, this is compatible with recent data (Pong and Levine 1976, Olsen and Ahnfelt-Ronne 1976), demonstrating PGE synthesis both in outer and inner cortex in dog. It should be remembered that the hydrogen washout method allow repeated measurements of local peritubular blood flow whereas the microsphere method measure glomerular blood flow.

In conclusion Pretreatment with indomethacin inhibit renal vasodilation in the early period of bleeding both in outer and inner cortex, indicating a temporary effect of PGE in all parts of cortex under hypotension.

While it is well known that the concentration of several vasoconstrictors in arterial blood increases radically during HH the present results suggest that their effect on the kidney is effectively but transiently counteracted by PGE in all parts of the cortex.

The nerves were stimulated with rectangular pulses of 2 ms duration with an intensity 5 times greater than that usually required for vagus nerve twitching (supramaximal stimulation). The preparations were stimulated at 1, 2, 5 and 10 Hz for 10 min at room temperature (21–23°C).

Assay of H MTLS after stimulation of the phrenic nerve-diaphragm preparation via the phrenic nerve. At the end of the stimulation period, the tissue preparations were frozen with liquid nitrogen and then homogenized in 5 ml of 0.1 M glycylglycine containing 12.5 mg/ml sodium-tetraphenylborate, extracted, chromatographed and measured as described in previous paper (Waldenström, in press). Measurements were also made of the perfusate fluid only (5) In 5 experiments, the non-innervated parts of the diaphragm muscle were immediately cut off after the stimulation as described by Potter (1970) hereafter MTLS as assayed as above.

Effect of thiamine on muscular contractions and on the formation of MTLS. The effect of different thiamine concentrations (10^{-4} – $5 \cdot 10^{-4}$ – 10^{-3} – $5 \cdot 10^{-3}$ M) on the muscular contractions were studied with the phrenic nerve as stimulated at frequency of 1 Hz. The muscular contractions were registered on Grass Polygraph. A possible dose-dependent relationship between impulse-frequency (1 and 5 Hz) and the formation of MTLS as investigated in the presence of thiamine at dose ($5 \cdot 10^{-3}$ M) which blocked the muscular contractions.

Effect of d-tubocurarine on the formation of MTLS. The effect of d-tubocurarine in different doses of 0.6 and 1 mg/l on the formation of MTLS in the phrenic nerve-diaphragm preparation as investigated. Stimulation was carried out at 5 Hz.

Release of 35 S-thiamine into the bath after stimulation of the phrenic nerve. 200 μ Cl of 35 S-thiamine is given to the rats 24 h before the experiments. The phrenic nerve-diaphragm preparation was dissected as described above. It was necessary to keep the muscle preparations in 1 litre of bath medium for 3 h to minimize the effect of radioactive thiamine from the muscle. Stimulation (1, 2 and 5 Hz for 10 min) as carried out in the presence of d-tubocurarine (12 mg/l). This dose is twice that required to inhibit muscle contractions due to phrenic nerve stimulation. The stimulation as followed by rest period of 10 min. Thereafter the procedure was repeated twice. The bath medium as changed after each stimulation and rest period. The bath medium (5 ml) as added to 10 ml of Instagel[®] and the 35 S-radioactivity as counted on liquid scintillation spectrometer. In 4 experiments the radioactivity of the baths as analyzed for identity with thiamine using ion pair extraction and paper chromatography as described earlier (Waldenström, in press).

Formation of MTLS in chopped denervated diaphragm. The left hemidiaphragm as deservated under ether anaesthesia by cutting the left phrenic nerve through an incision in the anterior chest wall. After 21 days, the right and left diaphragms were taken out, chopped finely with scissors and placed in 2 ml of Tyrode solution containing 40 μ Cl of 3 H-ACh (0.16 μ mol) or 40 μ Cl of 3 H-ACh (0.16 μ mol) or 40 μ Cl of 3 H-choline (0.16 μ mol) and thiamine (10^{-3} M final concn). After 3 min the tissue was extracted and chromatographed as described above. The number of experiments as shown in Table 1. The radioactivity as calculated as the percentage of the total added radioactivity per gram in weight of tissue (an innervated hemidiaphragm weighed about 1.5 g and deservated about 0.5 g).

Inhibition of nerve-induced contractions of the guinea-pig ileum by thiamine. Segments of terminal guinea-pig ileum as suspended in oxygenated Tyrode solution. The muscle contractions were registered on soaked drum. The effects of nicotine (ED_{50}) and ACh (ED_{50}) on the contraction of the isolated ileum were compared in the presence and absence of hexamethonium and thiamine. The effect of thiamine (300 μ M) on adrenaline, histamine and serotonin-induced contractions or relaxation was also measured. Adrenaline, histamine and serotonin were used in concentrations that gave response half as 50% of the maximal one. Thiamine as added 1 min before the substance to be tested. Every substance was tested at least 5 times. The number of experiments as shown in Fig. 3.

Addition of synthetic C2-methyl thiamine to homogenate of diaphragm. 2-methylthiamine was synthesized as described earlier (Waldenström, in press). 0.25 μ mol of 2-methylthiamine was added to homogenate of diaphragm (0.69 g wet weight) in 5 ml of bath medium. 20 min later the mixture was extracted as described above (x 6). Methylthiamine as assayed by thin layer chromatography as described earlier (Waldenström, in press). The spots were developed by Dragendorff reagent.

Materials

Choline chloride, ($N-(C^3H_9)$) (3 Cl/methyl- acetylcholine chloride, ($N-(C^3H_9)$) (30 mCl/mmol) and 35 S-thiamine hydrochloride (130 mCl/mmol) were obtained from the Radiochemical Centre, Amersham, England. Thiamine hydrochloride, acetone, hexamethonium bromide, carnitine sulphate and d-tubocurarine chloride were obtained from Sigma Chemical Company. Other chemicals were of the highest grade possible and obtained from usual commercial sources.

Release of Thiamine and Formation of a Methylthiamine-Like Substance in the Phrenic Nerve-Diaphragm Preparation of the Rat

By

LENNART WALDENLIND

Received 3 February 1977

Abstract

WALDENLIND L. *Release of thiamine and formation of a methylthiamine-like substance in the phrenic nerve-diaphragm preparation of the rat* Acta physiol. scand. 1977 101 22-27

An increased formation of a radioactive methylthiamine like substance (MTLS) in the end plate region of the rat diaphragm muscle and a release of radioactivity chromatographed as thiamine into the bath medium after a subcutaneous injection of ^{35}S -thiamine were found after stimulation of the phrenic nerve. There was also an increased formation of the radioactive methylthiamine-like substance in the denervated diaphragm preparation incubated with radioactive acetylcholine (ACh) in comparison with the associated diaphragm.

The formation of a methylthiamine-like substance (MTLS) in nerve tissue has recently been described (Waldenlind, in press). In the previous publication MTLS was designed "compound A". The MTLS was characterized by ion pair extraction and subsequent chromatography after labelling the nervous tissue with H -choline or ^{35}S -thiamine. It was found that the formation of radioactive MTLS was correlated with the formation of radioactive ACh and not with phospholipid formation or with the intermediary metabolism of choline.

To investigate whether or not MTLS was formed during cholinergic nerve stimulation—either in the nerve or in the innervated organ—the stimulated and denervated phrenic nerve-diaphragm preparation was used in the present study. In addition, the release of radioactivity chromatographed as thiamine into the bath fluid was measured. One possibility is that MTLS may in part be formed postsynaptically from released thiamine.

The action of thiamine on the function of different transmitter receptors was studied on the guinea-pig ileum preparation.

Experimental

Animals. Male Sprague-Dawley rats (100-150 g) were used. In some expts. the distal part of the guinea-pig ileum preparation was used.

Muscle preparations. The phrenic nerve-diaphragm preparations were set up according to Potter (1970). $1\text{ }\mu\text{M}$ of thiamine-hydrochloride and $1\text{ }\mu\text{C}$ of H -choline ($30\text{ }\mu\text{M}$ final conc.) were added to the bath fluid.

The nerves were stimulated with rectangular pulses of 2 ms duration with an intensity 5 times greater than that actually required for vigorous muscle twitching (supramaximal stimulation). The preparations were stimulated at 1, 2, 5 and 10 Hz for 10 min at room temperature (21–25°C).

Assay of H-MTLS after stimulation of the phrenic nerve-diaphragm preparation on the phrenic nerve. At the end of the stimulation period, the tissue preparations were frozen with liquid nitrogen and then homogenized in 5 ml ethyl alcohol containing 12.5 mg/ml sodium-tetraphenylboron, extracted, chromatographed and measured as described in previous paper (Waldenland, in press). Microsamples were also made of the perfused fluid only (5). In 5 expts. the non-stimulated parts of the diaphragm muscle are immediately cut off after the stimulation as described by Potter (1970) hereafter MTLS as assayed as above.

Effect of thiamine on muscular contractions and on the formation of MTLS. The effect of different thiamine concentrations (10^{-4} – $5 \cdot 10^{-4}$ – 10^{-4} – $5 \cdot 10^{-4}$ M) on the muscular contractions were studied on the phrenic nerve as stimulated at frequency of 1 Hz. The muscular contractions were registered on Grass Polygraph. A possible dose-dependent relationship between stimulus-frequency (1 and 5 Hz) and the formation of MTLS was investigated in the presence of thiamine at dose ($5 \cdot 10^{-4}$ M) which blocked the muscular contractions.

Effect of d-tubocurarine on the formation of MTLS. The effect of d-tubocurarine in different doses of 0.4 and 12 mg/l on the formation of MTLS in the phrenic nerve-diaphragm preparation was investigated. Stimulation was carried out at 5 Hz.

Release of 14 C-thiamine into the bath after stimulation of the phrenic nerve. 200 μ Cl of 14 C-thiamine as given to the rats 24 h before the expts. The phrenic nerve-diaphragm preparation was denervated as described above. It was necessary to keep the muscle preparations in 1 litre of bath medium for 3 h to minimize the effect of radioactive thiamine from the muscle. Stimulation (1, 2 and 5 Hz for 10 min) as carried out in the presence of d-tubocurarine (10 mg/l). This dose is twice that required to inhibit muscle contraction due to phrenic nerve stimulation. The stimulation as followed by rest period of 10 min. Thereafter the procedure as repeated twice. The bath medium as changed after each stimulation and rest period. The bath medium (5 ml) as added to 10 ml of Instagel[®] and the 14 C-radioactivity as counted on liquid scintillation spectrometer. In 4 expts. the radioactivity of the bath as analyzed for identity with thiamine using ion pair extraction and paper chromatography as described earlier (Waldenland, in press).

Formation of MTLS in chopped denervated diaphragms. The left hemidiaphragm as denervated under ether anaesthesia by cutting the left phrenic nerve through an incision in the anterior chest wall. After 1 day, the right and left diaphragms were taken out, chopped finely with scissors and placed in 2 ml of Tyrode solution containing 40 μ Cl of 3 H-ACH (0.16 μ mol) or 40 μ Cl of H-ACH (0.16 μ mol) or 40 μ Cl of 14 C-thiamine (0.16 μ mol) and thiamine (10^{-4} M final conc.). After 3 min the tissue as extracted and chromatographed as described above. The number of expts. is shown in Table 1. The radioactivity as calculated as the percentage of the total added radioactivity per gram wet weight of tissue (an averaged hemidiaphragm weighed about 1.5 g and denervated about 0.5 g).

Inhibition of nicotine-induced contractions of the guinea-pig diaphragm by thiamine. Segments of ventral guinea-pig diaphragm were suspended in oxygenated Tyrode solution. The muscle contractions were registered on a scaled drum. The effects of nicotine (ED₅₀) and ACh (ED₅₀) on the contractions of the isolated diaphragm were compared in the presence and absence of betanethonium and thiamine. The effect of thiamine (200 μ M) on adrenaline, histamine and acetylcholine-induced contractions or relaxation as also measured. Adrenaline, histamine and acetylcholine were used in concentrations that gave response which was 50% of the maximal one. Thiamine as added 1 min before the substance to be tested. Every substance was tested at least 5 times. The number of experiments is shown in Fig. 3.

Addition of synthetic C2-methyl thiamine to homogenate of diaphragm. 2-methylthiamine was synthesized as described earlier (Waldenland, in press). 0.5 μ mol of 2-methylthiamine as added to homogenate of diaphragm (0.49 g wet weight) in 5 ml of bath medium. 20 min later the mixture was extracted as described above (4). Methylthiamine was assayed by thin layer chromatography as described earlier (Waldenland, in press). The spots were developed by Dragendorff reagent.

Materials

Choline chloride, (N-(C³H₇)₄) (1 G/mmol), acetylcholine chloride, (N-(C³H₇)₄) (220 mC₁₀/mmol) and 14 C-thiamine hydrochloride (150 mC₁₀/mmol) are obtained from the Radiochemical Centre, Amersham, England. Thiamine hydrochloride, nicotine, betanethonium bromide, caerule sulphate and d-tubocurarine chloride were obtained from Sigma Chemical Company. Other chemicals were of the purest grade possible and obtained from usual commercial sources.

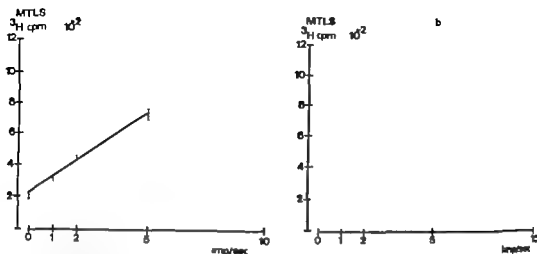


Fig. 1 Formation of MTLs in the phrenic nerve-diaphragm preparation after stimulation of the phrenic nerve. Stimulation was carried out in the presence of unchanged bath medium (a) in the presence of 10^{-4} M thiamine (b) and in the presence of d-tubocurarine (c). The regression lines in (a) and (c) were constructed using the method of least squares. The slopes of the curves were significantly different from zero. In (b) the slope of the calculated regression line was not statistically different from zero. a) MTLs (cpm) in unstimulated and stimulated preparations (1, 2 and 5 Hz). The stimulation at 10 Hz was not included in the calculation of the regression line. 10 expts. were carried out at each stimulation frequency. b) MTLs after stimulation in the presence of a high dose of thiamine. 8 or 9 expts. were carried out at each stimulation frequency. c) MTLs after stimulation (5 Hz) in the presence of different doses of d-tubocurarine. 10 expts. were carried out at each concentration.

Results

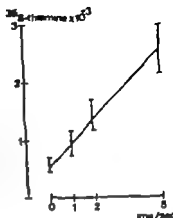
Increase of 3 H MTLs in the end-plate regions after stimulation of the phrenic nerve-diaphragm preparation. When the phrenic nerve was stimulated there was a stimulus-dependent increase of extractable 3 H MTLs from the diaphragms up to 5 Hz (Fig. 1 a). No measurable MTLs was formed in the muscle outside the end-plate region. No MTLs could be extracted from the bath medium ($n = 5$).

Effect of thiamine on muscular contractions and on the formation of MTLs. Thiamine at a concentration of $1-5 \times 10^{-4}$ M inhibited the contraction of the diaphragm. The inhibition was dose-dependent. When the muscle was paralyzed by thiamine (10^{-4} M) no frequency dependent formation of MTLs was observed (Fig. 1 b).

Effect of d-tubocurarine. d-Tubocurarine inhibited the formation of the MTLs in a dose-dependent manner (Fig. 1 c).

Release of 3 S-thiamine into the baths after stimulation of the phrenic nerve. A stimulus-dependent release of radioactivity chromatographed as thiamine into the bath medium was

Fig. 2 Release of 35 S-thiamine into the bath medium when the phrenic nerve-diaphragm preparation was stimulated via the phrenic nerve in the presence of *d*-tubocurarine (1 mg/l). The relative between stimulation frequency and the release of 35 S-thiamine is shown by the linear regression line calculated by the method of least squares. The slope of the regression line is significantly different from zero. The standard deviation for each stimulation frequency shown in the figure (4-6).



observed when the phrenic nerve was stimulated in the presence of *d*-tubocurarine in a dose twice as high as that which inhibited the muscle contractions (Fig. 2). In the 4 expts. where the bath medium was extracted and chromatographed as before (Waldenlind, in press). 85-94% of the radioactivity was measured as thiamine. Therefore the 35 S-radioactivity should be a good indication of the thiamine content.

Formation of MTLS from denervated diaphragm (chopped tissue) When denervated rat diaphragms were incubated with radioactive ACh, significantly more MTLS was found in denervated than in innervated tissue (Table 1). Incubation with choline alone gave no detectable formation of MTLS.

Inhibition by thiamine of the effect of nicotine on the guinea-pig ileum. The stimulatory action of nicotine (ED_{50} 10-30 μ M) on the guinea-pig ileum was inhibited dose-dependently by thiamine (40-300 μ M) (Fig. 3). The inhibition lasted at least 60 min. Removal of thiamine by changing the bath fluid immediately restored the sensitivity of the intestine to nicotine. Thiamine did not change the response of the intestine to ACh, histamine, serotonin or adrenaline. The effect of thiamine was found to be similar to that of hexamethonium—blockage of the response to nicotine but not to that of ACh.

Addition of C2-methylthiamine to homogenate of diaphragm No C2-methylthiamine could be extracted from the homogenate of the diaphragm muscle after the addition of synthetic C2-methylthiamine (6). Only thiamine could be detected in the chromatograms.

Table 1 Comparison of the formation of methylthiamine-like substance after incubation of chopped homogenate of innervated and denervated diaphragm in the presence of thiamine and 3 H-ACh. The values are given as percentage of the totally added radioactivity per g wet weight of tissue (an innervated hemidiaphragm weighs about 1.5 g and denervated about 0.5 g). The mean and standard deviations are given in the table.

	Innervated	Denervated
Amount of methylthiamine	0.079 \pm 0.013 (n=10)	0.44 \pm 0.054 (n=10)

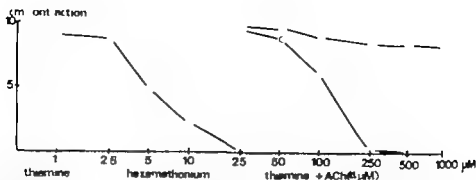


Fig. 3 Dose-dependent inhibition by hexamethonium and thiamine of nicotine-induced contraction of the intestine is shown in the two sigmoid curves. The third curve shows the effect of thiamine on ACh-induced contractions. The response to ACh ($4 \mu\text{M}$) showed a small decrease at thiamine concentrations between 25 and $100 \mu\text{M}$ of thiamine and was therefore not further decreased at least to a concentration of 1 mM . All curves were reproducible when tested 5 times.

Discussion

In a previous paper (Waldenlind, *in press*) the formation of MTLS *in vivo* especially in cat ventral roots, after a H-choline injection locally into the spinal cord has been described. The synthesis of ACh showed a time-curve similar to that of MTLS formation in contrast to the formation of phospholipids. Betaine, the oxidation product of choline which is metabolised in the intermediary metabolism by donating methyl groups, could not act as a precursor of MTLS. For various reasons it was suggested that the synthesis of MTLS may reflect nicotinic receptor activity. This hypothesis was to be further tested by using a physiological preparation such as the phrenic nerve-diaphragm preparation. By stimulating the phrenic nerve it was thought that the cholinergic transmission was activated. The production of MTLS increased as can be seen in Fig. 1 a. It was, however, important to freeze the preparation immediately in liquid nitrogen—otherwise the transient increase of MTLS could not be calculated from the results. The MTLS was also concentrated to the end plate region of the diaphragm muscle, which indicates a relation to nerve terminal structures. No MTLS was detected in the nerve-free parts of the muscle.

By denervating hemidiaphragms it was possible to compare the amount of MTLS formed in chopped innervated and denervated diaphragms. The amount of MTLS was about 5.6 times higher in the denervated diaphragms than in the innervated diaphragms if the calculations were based on the weight of wet tissue and about twice of the results were expressed per hemidiaphragm (the innervated diaphragms were heavier). As denervation is known to increase the number of extrajunctional ACh-receptors (Axelsson and Thesleff 1957), the increased amounts of MTLS could be related to this change. Conclusions must however be drawn with care since the muscle metabolism is probably changed by the denervation procedure.

Thiamine in a dose which blocked the muscular contractions due to phrenic nerve stimulation gave no stimulus-dependent increase of MTLS (Fig. 1 b). Instead the basal levels of MTLS were elevated at all stimulation frequencies suggesting that the formation of MTLS was stimulated. In contrast α -tubocurarine inhibited the formation of MTLS in a dose-

dependent manner (Fig. 1 c). This was the same dose-range as that which blocked the muscular contractions.

In addition, a release of 35 S-thiamine was found from stimulated curarized phrenic nerve diaphragm preparations. An attractive idea is that the MTLs was formed from released 35 S-thiamine. This suggestion is, at least on the basis of the present expts., only a speculation. An increased release of thiamine from the phrenic nerve has been described by Minz (1938). A microbial method for thiamine assay was used.

Thiamine inhibits the diaphragm contractions elicited by phrenic nerve stimulated and the nicotine-induced response of the guinea-pig ileum (in 50-100 μ M concentration). The effect on the guinea-pig ileum was a specific inhibition of the nicotine-induced contractions. The effect of other amines tried were not changed. Thus it seems that thiamine has a specific affinity to structures mediating the nicotine response. Similar results have been described by Uvna and Pick (1944).

The results reported in the present paper support the hypothesis that activation of nicotine receptors leads to formation of MTLs. It is, however, possible that MTLs is related by another mechanism to nerve metabolism. The expts. with the phrenic nerve-diaphragm preparation do not show whether the synthesis of MTLs occurs presynaptically or post synaptically. The "cholinergic receptors" in the denervated diaphragm are not identical with the postsynaptic ones and, besides, reinnervation might have occurred to some extent.

In order to try to rule out the possibility that MTLs is an artefact of the procedure used, blank extractions and chromatograms with radioactive choline and ACh were made as had been described in a previous paper (Waldenlind, in press). MTLs could only be found if choline was allowed to be metabolized in an unhomogenized tissue. Ion-pair extraction from homogenized tissue to which choline or ACh had been added yielded no MTLs. Despite these controls, external factors influencing the extraction or chromatography and leading to an artefact described as MTLs may have been overlooked, although this must be considered improbable.

A role for thiamine in neuroconduction has been proposed by v. Muralt (1962) and Cooper (1968). They found an increased release of thiamine from stimulated nerves. In future investigations, the importance of thiamine for nicotinic receptor activity will be investigated.

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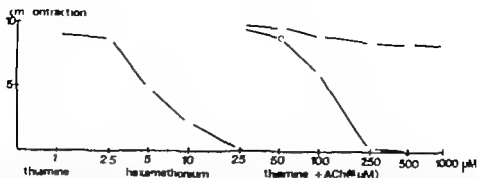


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Plasma Protein Dynamics: Albumin and IgG Capillary Permeability, Extravascular Movement and Regional Blood Flow in Unanesthetized Rabbits

By

ANDERS BILL

Received 11 February 1977

Abstract

BILL, A. *Plasma protein dynamics. Albumin and IgG capillary permeability, extravascular movement and regional blood flow in unanesthetized rabbits.* Acta physiol. scand. 1977 101: 28-42.

The turnover rate constants for extravascular albumin and immunoglobulin-G (IgG) and the turnover rates and extravascular spaces for the proteins and regional blood flow were determined in different tissues in unanesthetized rabbits. The turnover rate constants (percent per minute) for extravascular albumin were 1.9 in the kidney cortex and 1.4 in the kidney medulla, about 0.8-1.4 in the lung, small intestine, choroid plexus and heart muscle and about 0.2-0.6 in the skeletal muscle, stomach wall and gall bladder. The turnover rate constants for IgG were similar or lower. The plasma equivalent extravascular albumin space was 145 μ l/g in the kidney medulla, about 100 μ l/g in the gall bladder, heart muscle, choroid plexus and small intestine, 30-70 μ l/g in the kidney cortex, lung, stomach wall and triceps muscle. The extravascular IgG spaces were almost smaller. In the liver, spleen and adrenals where extravascular spaces could not be determined, the ratios total IgG space/total albumin space were about 1.3-1.5. Extraction of albumin defined as turnover rate of extravascular albumin/regional plasma flow could not be determined in the liver, spleen and adrenals. It was zero in the brain cortex, about 0.001 in the lung and around 0.01-0.04 in most other tissues. The results suggest that in the walls of the capillaries of the kidney cortex, stomach wall, skeletal muscle and gall bladder there was convection of the proteins with pinocytosis and/or through large pores, diffusion playing little role. The albumin and the IgG passed through the trachea and were drained essentially by bulk flow. In the heart muscle, choroid plexus, lung and small intestine there seemed to be some diffusion of albumin through the capillary wall in addition to convection. Accumulation of IgG in the liver, spleen and adrenals was probably caused by adsorption to cells belonging to the RES.

Key words: Albumin, immunoglobulin-G, capillary permeability, tissue fluid, protein, protein extraction.

In most tissues there is a leakage of protein from the capillaries into the tissue fluid and a drainage of protein from the tissue by the lymph vessels. The movement of protein from the capillaries into the tissue fluid and back into the general circulation by way of the lymph vessels or other routes is of importance for the filtration of fluid from the capillaries. It is also of importance for many other mechanisms—such as the defence against antigens.

substances, the replacement of vascular proteins after blood loss, and the distribution of fatty acids, many hormones, vitamins and drugs.

Studies on lymph flow and lymph composition have contributed greatly to the understanding of several important aspects of the extravascular exchange of macromolecules in anesthetized animals (Grotte 1956, Garlick and Renkin 1970, Johnson and Richardson 1974) and in heart-lung preparations (Arturson, Groth and Grotte 1972).

Grotte's (1956) studies on the movement of dextrans of different molecular weights from plasma to lymph indicated that there are two kinds of functional pores in the walls of the capillaries: small pores with a diameter of about 8 nm and large pores or leaks with diameters of 24–70 nm (see also Pappenheimer 1970). The plasma proteins were thought to pass out through the large pores. The results of later estimates of pore sizes have varied somewhat but the two pore-size hypothesis has gained increasing support (Michel 1972, Crone 1974).

In some tissues, e.g. liver and spleen, the large pores are easily identified: there are gaps in the capillary walls which are even larger than 70 nm (Majno 1966). In other tissues the conditions are less clear. In the capillaries of skeletal, heart and smooth muscle, no clearcut candidates for the role as real large pores have been found. This may be due to a very low frequency of large pores or actual pores may be absent. In such tissues pinocytotic vesicles, which have been observed to transport molecules as large as ferritin with a diameter of 11 nm, may very well be the only representative for the large pore system (Karnovsky 1962, Renkin 1964, Waine 1965). Strong evidence for this hypothesis was presented by Garlick and Renkin (1970). In other organs, e.g. the small intestine, parts of the endothelial cells of the capillaries are very thin and in these places there are large numbers of fenestrations. These fenestrations have a diameter of about 40 nm, nicely within the limits for the large pores predicted by Grotte. However in electron micrographs these fenestrations are often closed by a thin membrane. It is not clear if this membrane is present in all fenestrations or just a proportion under in vivo conditions, and it is possible in fact that there are differences between organs in this respect. Studies with marker molecules have indicated that macromolecules may pass through fenestrae as well as with pinocytosis (Simionescu, Simionescu and Palade 1972).

Elaborate classifications of capillaries take into account the ultrastructure of the endothelial cells, the basement membrane and the pericapillary investment (Bennett, Luft and Hampton 1959). But in this discussion capillaries will be referred to as gap capillaries and as fenestrated and continuous capillaries. Protein exchange taking place in venules and small veins will be included with that taking place through the capillary walls.

Lymph studies of course can give information only about the net outflow of macromolecules from the capillaries. A usual assumption was that there is little return of macromolecules into the capillaries within the tissues. This may be true for some tissues but return of protein into the general circulation within the tissue has been reported for the liver (Goresky 1970), the kidney (Swann 1960), and skeletal muscle (Perry and Garlick 1975). It has been argued (Cassidy-Smith, O'Donoghue and Crocker 1975) that the reabsorption of protein into the venous part of the capillaries may be a usual phenomenon in tissues with fenestrated capillaries.

Studies on the accumulation of labelled albumin in the various tissues of mice have been

reported by Friedman (1957) and Dewey (1959). Studer and Potchen (1971) have reported data for many tissues in rats and Owen and Triffitt (1976) have presented data for some tissues in rabbits. Such data are of great interest because they can be used to calculate the volumes of the extravascular albumin spaces and the rate of outflux of albumin from the capillaries. But they give no information about the mechanism for protein drainage from the tissues.

Previous communications (Bill 1968 a, b) reported on the accumulation of labelled albumin and gammaglobulin in some tissues by employing a double isotope method. These studies, centered on intraocular tissues, showed that simultaneously determined spaces may enable a more detailed analysis of the extravascular movement of proteins than studies on single proteins.

The purpose of this investigation was to extend the previous study to other tissues. The regional blood flow was determined in order to relate protein outflux from the capillaries to the plasma flow.

Methods

Albin rabbits of both sexes, weighing 2-3 kg each, were used in the determinations of the protein spaces and the regional blood flow. The animals were given pellets and water ad lib. before and during the expts and were kept at a room temperature of about 22°C. Each animal was free to move in a cage with a floor space of about 40 × 50 cm. Rabbits weighing 0.8-1.0 kg were used in the screening of the proteins.

1. Experiments with ^{125}I -albumin and ^{131}I -albumin

The animals were given albumin labelled with one iodine isotope in such a way that the concentration in the plasma was kept almost constant for a period of time varying from 15 min to 48 h (Bill 1968 b). Albumin labelled with the other iodine isotope was given for a shorter period of time in such a way that the plasma concentration of this albumin was also kept almost constant until the end of the expts. The animal was killed by a rapid injection of 60 mg/kg b wt. pentobarbital sodium followed by 3 ml saturated KCl.

2. Experiments with ^{125}I -albumin and ^{125}I -immunoglobulin G

The labelled proteins were injected i.v. Additional injections kept the plasma concentration of the two proteins almost constant.

In these expts. screened proteins were used except a few preliminary expts. The total injected doses were less than 10 mCi.

Labelled proteins. Human serum albumin labelled with ^{125}I or ^{131}I was obtained from AB Atomenergi, Studsvik. Rabbit serum albumin and IgG were purified by gel chromatography on Sephadex G 700 (Pharmacia, Uppsala) and labelled with ^{125}I or ^{131}I according to McFarlane. To remove iodine not bound to the protein, the proteins were subjected to gel chromatography on Sephadex G 25 immediately before use. The results obtained with the different labelled albumins indicated that there was a small fraction of the albumin that was eliminated very quickly from the blood stream by the kidneys. The proteins were screened in order to remove this probably slightly denatured protein. For screening the proteins were injected i.v. into small rabbits. The plasma obtained from these rabbits 30-60 min later was given to the animals in which the turnover rates were to be determined.

Assay. The radioactivity was determined by means of two-channel gamma-spectrometry. Precipitation with 20% trichloroacetic acid was used to determine the amount of iodine not bound to the protein in the blood and tissue samples (for details see Bill 1968 b). Plasma equivalent albumin and IgG spaces were calculated as in the previous study.

3. Regional blood flow

The regional blood flow was determined in unanesthetized rabbits one day after the cannulation of the left heart ventricle for microsphere injection and one femoral artery for the collection of reference samples.

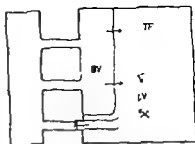


Fig 1 Model I for extravascular exchange of proteins as discussed under "Theory". BV represents the blood vessels of the tissue; TF—tissue fluid; LV—the lymph vessels. Arrows indicate direction of protein fluxes.

The heart is cannulated via the left brachial artery. The spheres were 15 μ m carboxylated microspheres (Gibco Company), labelled with ^{59}Fe . The regional blood flow in $\text{g min}^{-1} \text{g}^{-1}$ as calculated as described elsewhere (Kjorvick, Allen and Bell 1976). The plasma flow in $\text{ml min}^{-1} \text{g}^{-1}$ as calculated as blood flow $\times (100 \text{ haematocrit}) / (100 - 100)$.

Theory

The results of the previous study (Bell 1966) and those of the present experiments indicate that extravascular plasma protein exchange varies from one tissue to another. A rather simple model seems to describe the situation in some tissues, but in others the results are somewhat ambiguous.

Fig 1 shows the simple model I. The pathways between BV and TF may be pericyclic vesicles operating in one direction only or actual pores in which outward filtration is so rapid that return of the macromolecules by diffusion is hindered (see Bell 1966). The membrane separating TF from LV is assumed not to discriminate between the albumin and IgG, both enter LV by the bulk flow of the tissue fluid. In this model LV may then be included in TF.

If radioactive albumin is added to the general circulation and the plasma concentration is kept constant, compartment BV will reach steady state concentration of the labelled albumin short time after. Albumin will also enter TF. Diffusion of albumin in the tissue and the movement of the tissue fluid will give an apparent "mixing" of the tissue fluid. Labelled albumin is therefore also lost from the TF by way of the lymph vessels almost from the start. After some time steady state is reached. The specific activity of the albumin in the tissue fluid then is the same as that in the plasma. The replacement of the unlabelled albumin originally present in the TF by albumin having the same specific activity as the plasma is described by the equation:

$$EAV = E_{\text{max}} e^{-k_{\text{av}} t}$$

here EAV—the apparent volume per g tissue of unlabelled albumin left in the tissue at time t , and E_{max} is the plasma equivalent extravascular albumin space per g tissue—defined as $\text{ml albumin/g tissue}$ divided by $\text{ml albumin/ml plasma}$. This volume is equal to the steady state extravascular plasma equivalent albumin space measured with radioactive albumin, E_{max} . The k_{av} —the turnover rate constant for albumin in TF. EAV at all times equals $E_{\text{max}} - E_{\text{av}}$, here E_{max} is the steady state plasma equivalent albumin space per g tissue and E_{av} the apparent plasma equivalent albumin space at time t , as determined with labelled albumin.

$$EAV = E_{\text{max}} - E_{\text{av}} = E_{\text{max}} e^{-k_{\text{av}} t}$$

In a semilogarithmic plot of $E_{\text{max}} - E_{\text{av}}$ versus time, line of best fit may be drawn, and the $T_{1/2}$ can be read and the turnover rate constant for albumin, k_{av} , can be calculated: $k_{\text{av}} = 0.693/T_{1/2}$. The E_{max} equals the intercept of the line with the ordinate. The plasma volume (PBV) remaining in BV after the death of the animal and the destruction can be calculated as

$$PBV = E_{\text{max}} - E_{\text{av}}$$

See Bell (1966) and Fig 4.

The same considerations as above can be made for IgG having the turnover rate constant k_{IgG} in TF. On the microspheres made, k_{av} and k_{IgG} should be very similar in the same tissue. This is true since the turnover rate constants all differ only if there is barrier in the tissue draining the proteins that restrict

the movement of the IgG more than that of the albumin or if diffusion plays a great role in the elution of the proteins.

The sieving in the capillary walls makes the steady state albumin space in TF larger than the corresponding steady state IgG space—as a consequence the turnover rate of albumin in TF defined as $k_{app} A$ will be larger than the IgG turnover rate determined analogously.

Since model 1 assumed no return of protein into the blood vessels, the turnover rate for albumin equals the apparent leakage of the plasma equivalent fluid (with respect to albumin) out of the capillaries. It is equal to the drainage of such fluid from the tissue by the lymph vessels. The turnover rate for IgG is logically equal to the apparent leakage of the plasma equivalent (with respect to IgG) fluid out of the capillaries and its drainage by the lymph vessels. The ratio turnover rate for IgG/the turnover rate for albumin gives a measure of the restriction to outflow of IgG as compared to albumin in the capillary wall. There is one source of ambiguity: a return of plasma proteins into the capillaries without discrimination between albumin and IgG cannot be distinguished from bulk drainage into the lymph vessels.

Model 2 which covers several alternatives differs from model 1 in that the extravascular IgG has a less turnover rate constant than the albumin. Some mechanisms that may give a real or an apparent slower hindrance for the IgG are: a) elimination of protein from the tissue by diffusion back into the capillaries through pores or movement back into the capillaries with size discriminating pinocytosis, b) flow of the fluid through a barrier in the tissue or the lymph capillary wall that is more permeable to the albumin than to the IgG, c) adsorption of the IgG to some tissue component and d) movement by diffusion over relatively large distances that will make IgG lag due to its lower diffusion coefficient.

The turnover rate for albumin again gives the apparent leakage of albumin out of the capillaries but its net outflow may be less because of the albumin returning into the capillaries. The same reasoning holds for the IgG. The ratio turnover rate for IgG/turnover rate for albumin gives a measure of the relative restriction in the capillary wall to IgG compared to that of the albumin.

It would ideally be possible to decide if model 2 behaviour is due to a return of the albumin by diffusion and/or pinocytosis into the capillaries (Model 2 A) or to some mechanism resulting in marked filter effect (Model 2 B). In the former case one can expect the turnover rate of albumin to be much faster than that of IgG. In the latter case one can expect a normal relationship between the turnover rates but a large IgG space as compared to the albumin space.

Results

Albumin spaces

In the expts. with I -albumin and ^{51}I -albumin steady concentrations in the plasma of the two labelled proteins were maintained for two different periods of time. As a consequence 2 values were obtained for the plasma equivalent albumin spaces in each sample. In the expt. with albumin and IgG only one value per sample was obtained for the plasma equivalent albumin space. The values determined for the albumin space each time were pooled for each tissue. The mean values, usually calculated from 4 samples of each tissue in each of 10–12 rabbits, were plotted against time, Fig. 2 A.

In several expts. with unscreened human and rabbit serum albumin it was found that the kidney cortex had a larger 15 min albumin space than the 6 h space determined in the same animal. This result was seen irrespective of the albumin used to determine the 15 min space. This indicated that both ^{125}I -albumin and ^{51}I -albumin contained a small fraction of denatured labelled protein that was rapidly eliminated by the kidneys. In order to eliminate artifacts caused by such denatured albumin, S_{at} at different times was determined with screened albumin in the kidney and some of the other tissues. In this series of experiments the 5 and 30 min space was determined in five animals and the difference was calculated for each tissue. Other space pairs determined were the 30 and 60 min spaces, the 1 and 4 h spaces and the 4 and 8 h spaces. The 4 h values and the differences observed were used to construct S_{at} versus time plots (Fig. 2 B). Usually 4 samples were taken from each tissue at

Fig. 2A The apparent plasma volume albumin space in lung, skin and skeletal muscle at different times after the injection of labeled albumin. Mean values and standard errors are shown.

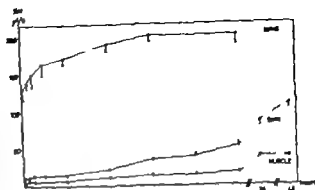
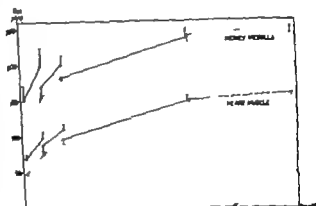


Fig. 2B Observed values for the plasma equivalent albumin space at different times—solid symbols, and values calculated from the 4 hour space and the differences observed—open symbols.



5 animals. With screened albumin the S_{app} increased with time—as expected—in the kidneys. For the other tissues investigated, the results obtained with screened albumin were similar to those obtained with the unscreened. In semilogarithmic plots of $S_{app} - S_{app}$ versus time there was a much better fit between the line and the points with the second method than with the first. The reason for this was no doubt that variations in the blood volume between animals did not affect the results of the second method.

Table I shows the values for the rapidly exchanging plasma equivalent spaces which were assumed to be intravascular and the volumes of the extravascular spaces calculated from the semilogarithmic plots, the turnover rate constants for albumin, and the turnover rate for albumin in the tissues. The values shown were obtained both with unscreened and screened albumin for the skin, the triceps muscle and the lung. For the other tissues the values were calculated from the expts. with screened albumin only. In the brain, liver, spleen, bone marrow and adrenals the increase with time in the calculated albumin space from 5 min and onward was less than 10%. No meaningful values could be calculated for the turnover rates and the extravascular spaces in these tissues.

For the skin no turnover rate constant could be determined. From the appearance of the

TABLE I Data for albumin spaces

Tissue	Fast albumin space $\mu\text{l g}^{-1}$	Slow albumin space, S_{slow} $\mu\text{l g}^{-1}$	k_{alb} min^{-1}	$k_{\text{alb}} S_{\text{slow}}$ $\mu\text{l min}^{-1} \text{g}^{-1}$
Kidney medulla	90	145	0.014	2.03
Heart muscle (left ventricle wall)	44	113	0.011	1.26
Kidney cortex	59	64	0.019	1.20
Small intestine	21	84	0.013	1.09
Choroid plexus	137	93	0.0096	0.89
Gall bladder	47	107	0.0060	0.64
Lung	124	72	0.0083	0.58
Stomach wall (corpus)	20	58	0.0030	0.17
Skin	11	—	—	0.066
Triceps muscle	4	30	0.0017	0.051

plot for the apparent albumin space versus time, it seemed reasonable to assume that the 2 hours following the first injection of labelled albumin, only a negligible amount of extravascular labelled protein occurred in this tissue—the space increased almost linearly. An approximative value for the turnover rate of the albumin could then be calculated by dividing the difference between the 2 and 5 min spaces by the variance in time.

Albumin and IgG spaces

In these expts. the plasma concentration of the two labelled proteins was kept almost constant from the beginning of the expts. until the animal was killed. The apparent equivalent albumin and IgG spaces, S_{alb} and S_{IgG} , respectively, were calculated at different times. For most tissues there was such variability in the spaces that the turnover constants and turnover rates could not be determined with accuracy from the space data. In each expt. for each tissue, S_{IgG} was divided by S_{alb} to obtain the IgG/albumin space ratio, SR. The value for the space ratio each time varied moderately for each tissue. Fig. 3 shows plots of IgG/albumin space ratios versus time.

The space ratios were used to construct curves describing the accumulation in the tissue of labelled IgG. For this purpose values for the idealized S_{alb} denoted $S_{\text{alb}}^{\text{ideal}}$ were calculated from the line in the semilogarithmic plots of $S_{\text{slow}} - S_{\text{alb}}$ versus time. To obtain $S_{\text{IgG}}^{\text{ideal}}$ the idealized value was multiplied by the pertinent SR denoted SR.

$$S_{\text{IgG}}^{\text{ideal}} = \text{SR} \cdot S_{\text{alb}}^{\text{ideal}}$$

For each tissue $S_{\text{slow}} - S_{\text{IgG}}^{\text{ideal}}$ was plotted against time and the turnover rate constant k_{IgG} was calculated. The vascular space and the apparent extravascular space were calculated as for the albumin spaces. Fig. 4 shows the semilogarithmic plot for heart muscle. Table II III and IV show the results. Space ratios at 5 and 30 min and 24 h were based on determinations of the means of 4 samples from 5 animals ($n=5$). Each of the ratios at 15 min, 2, 4, 6 and 10 h was based on 4 determinations in 12 animals. The whole choroid plexus was taken as one sample.

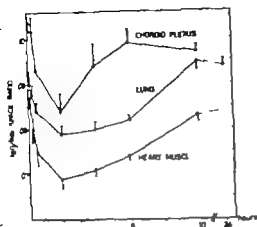


Fig. 3

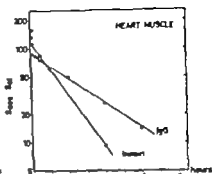


Fig. 4

Fig. 3 The IgG/albumin space ratio for different tissues was plotted against time. Mean values and standard errors are shown.

Fig. 4 A semi-logarithmic plot of the steady state albumin space versus the space at different times versus time. The line was drawn by eye and $T_{1/2}$ determined from the line. The intercept of the line with the space axis gives the apparent volume of the extravascular space. The difference between the intercept and the steady state space—curve—was the volume of the intravascular space in dead animals. The identical values for the albumin space at different times as determined using the line. This value multiplied by the pertinent space ratio gives the apparent volume of the IgG space at different times. The steady state plasma equivalent IgG space—open squares—shows the space at different times was also plotted versus time and the $T_{1/2}$ and apparent volumes of extravascular and intravascular spaces were determined as for the albumin.

Monoeponential curves seemed to describe the situation in all tissues both for the albumin and the IgG.

Regional blood flow protein extraction

Table V shows the values for the regional blood flow ($n = 10$) and albumin- and IgG-extractions. Extractions were calculated as turnover rate/regional plasma flow and expressed as percentages.

TABLE II Data for IgG spaces

Tissue	First IgG space $\mu\text{l g}^{-1}$	Slow IgG space, S_{system} $\mu\text{l g}^{-1}$	k_{IgG} min^{-1}	$k_{\text{IgG}} \frac{S_{\text{albumin}}}{S_{\text{IgG}}}$ $\mu\text{l g}^{-1} \text{min}^{-1} \text{g}^{-1}$
Kidney medulla	82	116	0.031	1.27
Heart muscle				
Left ventricle (all)	43	98	0.005	0.44
Kidney cortex	54	56	0.019	1.06
Small intestine	21	78	0.008	0.60
Choroid plexus	137	81	0.0040	0.36
Gall bladder	46	26	0.0060	0.32
Lung	134	68	0.0029	0.1
Stomach wall (corpus)	20	36	0.0030	0.11
Skin	11	—	—	0.090
Triceps muscle	4	15	0.0017	0.025

TABLE III Ratios and capillary ultrastructure

Tissue	Turnover rate ratio		Turnover rate constant ratio		Extravascular space ratio	Ultrastructure of capillaries
	$\frac{k_{IgG}}{k_{alb}}$	$\frac{S_{IgG\text{Oss}}}{S_{alb\text{Oss}}}$	$\frac{k_{IgG}}{k_{alb}}$	$\frac{S_{IgG\text{Oss}}}{S_{alb\text{Oss}}}$		
Kidney medulla	0.63		0.8		0.80	Fenestrated
Heart muscle (left ventricle wall)	0.35		0.5		0.77	Continuous
Kidney cortex	0.88		1.0		0.88	Fenestrated
Small intestine	0.55		0.6		0.93	Mainly fenestrated
Choroid plexus	0.40		0.4		0.87	Fenestrated
Gall bladder	0.81		1.0		0.80	Mainly fenestrated
Lung	0.36		0.4		0.94	Continuous
Stomach wall (corpus)	0.65		1.0		0.62	Mainly continuous
Skin	0.75		—		—	Continuous
Triceps muscle	0.49		1.0		0.49	Continuous

Discussion

In tissues where the extravascular albumin spaces were larger than the extravascular IgG spaces and the turnover rate constant for IgG/the turnover rate constant for albumin was about unity model 1 describes the situation. The albumin and IgG passed out of the capillaries by a process that restricted the movement of the IgG compared to that of albumin but the plasma proteins left the tissue essentially by bulk flow. Bulk drainage is easily envisaged as drainage with lymph but the possibility of pinocytosis without size discrimination back into the capillaries has to be kept in mind. The mechanism causing protein outflow from the capillaries is less clear. There are two main alternatives, filtration through relatively large pores where diffusion plays little role (see Winne 1965, Blake and Staub 1976) and movement with pinocytosis. And it is possible, of course, that the two mechanisms might operate in parallel. Table III shows that tissues with continuous capillaries as well as tissues with fenestrated capillaries belong to this group. The turnover rate of IgG/turnover rate of albumin was 0.49–0.88. Such rather variable sieving might result from convection through

TABLE IV Total plasma equivalent albumin and IgG spaces in tissues that had such rapid turnover of albumin that the tissue fluid that turnover rates could not be calculated. The mean values are calculated from data at different times (-30) Values for brain are also shown here. There was no indication for an extravascular protein space in the brain. IgG/albumin space ratios are also shown

Tissue	Albumin space		IgG space		$\frac{S_{IgG\text{Oss}}}{S_{alb\text{Oss}}}$
	S_{alb} $\mu\text{l g}^{-1}$		S_{IgG} $\mu\text{l g}^{-1}$		
Adrenals	65 \pm 2		85 \pm 2		1.30 \pm 0.02
Liver	116 \pm 4		179 \pm 8		1.54 \pm 0.06
Spleen	72 \pm 2		98 \pm 2		1.38 \pm 0.02
Bone marrow (from ribs)	124 \pm 5		127 \pm 5		1.03 \pm 0.01
Brain, cortex	52 \pm 0.2		52 \pm 0.2		1.00 \pm 0.01

TABLE V Regional blood flow, plasma flow and protein extraction

Tissue	Blood flow $\text{g min}^{-1} \text{g}^{-1}$ $\text{M} \pm \text{S.E.}$	Plasma flow $\text{ml min}^{-1} \text{g}^{-1}$	Albumin extraction	IgG extraction
<i>d.</i>	<i>d.</i>	<i>d.</i>	<i>d.</i>	<i>d.</i>
kidney cortex				
heart muscle				
left ventricle (all)	5.7 ± 0.8	3.6	0.036	0.012
kidney cortex	6.4 ± 0.3	4.0	0.030	0.027
small intestine	1.2 ± 0.3	0.7	0.156	0.086
choroid plexus	3.2 ± 0.4	3.3	0.027	0.011
gall bladder	2.2 ± 0.3	1.3	0.048	0.040
lung	<i>d.</i>	<i>d.</i>	<i>d.</i>	<i>d.</i>
bowel wall (coron)	1.95 ± 0.3	1.2	0.014	0.009
skin	0.20 ± 0.03	0.12	0.054	0.042
triceps muscle	0.20 ± 0.05	0.12	0.041	0.021

large real pores with average sizes varying from one tissue to another. If due entirely to pinocytosis one has to assume that there are different kinds of vesicles, some, P_{10} , transporting proteins with little size discrimination, others, P_{100} , causing marked sieving effects.

In a number of other tissues—again with continuous or fenestrated capillaries—the data cannot be explained by model 1. Here the turnover rate constant for albumin was higher than that for IgG. The ratio turnover rate of IgG/turnover rate of albumin was similar to that in the vascular beds described by model 1 or lower. So far model 2 A thus seems to describe the conditions in these tissues. But one has to consider also the extravascular space ratios. In all tissues in question the ratios were rather high. The question is then whether there is any indication for selective IgG accumulation in the tissues. A tentative answer to this question can be obtained by comparing the extravascular space ratios with known values for the lymph/plasma ratios for the two proteins. In dog hearts the albumin $C_{\text{lymph}}/C_{\text{plasma}}$ ratio is 0.74 and the corresponding value for globulin is 0.55 (see Courtois *et al.* 1974). The extravascular globulin/albumin space ratio expected if the tissue fluid had the same relative concentrations as the lymph then would be $0.55/0.74 = 0.74$. The observed value for the extravascular IgG/albumin space ratio was 0.77. Thus if it is assumed that IgG concentrations are roughly proportional to total globulin concentrations, model 2 A seems to describe the situation in heart muscle. In the small intestine the extravascular space ratio expected from corresponding data in dogs is about 0.8 and in cats 0.9 (see Courtois *et al.* 1974). This is in reasonable agreement with the observed value, 0.93.

In the lung a recent study in sheep (Blake and Staub 1976) indicates that the extravascular space ratio should be 0.65. Taylor *et al.* (1967) obtained results in adult dogs indicating a value of 0.70 (in pups about 1.0). The value observed here, 0.94, thus is far above that expected in adult animals suggesting that in this tissue one has to consider also model 2 B.

Capillary permeability

Table I and II show more than 20-fold differences in the turnover rates of albumin and IgG between the different tissues. The results are in apparent agreement with the traditional concept that the protein permeability in fenestrated capillaries is higher than that in the

continuous capillaries. And heart muscle is an exception in having continuous capillaries with a high protein permeability. However if extraction values are compared this suggestion becomes highly questionable. Extraction of albumin, E_a , and IgG was defined as the turnover rate of the protein in question/regional plasma flow. The outflux of albumin through a capillary bed into the tissue depends on the permeability of the vessels to albumin, P_a , and the surface of the capillaries, S , and the concentration of albumin in plasma, C_{ap} :

$$J = P S C_{ap}$$

The amount of albumin entering the capillary bed with the blood depends on the plasma flow Q and the plasma concentration of albumin, C_{ap} :

$$\text{Thus } E_a = \frac{P S C_{ap}}{Q C_{ap}} = \frac{P S}{Q}$$

The conditions for IgG are analogous. The extraction values, Table V, in a number of tissues were quite similar irrespective of differences in capillary ultrastructure, Table III. The plasma flow is related to the number of perfused capillaries and thereby to the capillary surface—which is the case if the average blood flow in the perfused capillaries is about the same in the different tissues—it turns out that the protein permeability of the capillaries in most tissues with continuous or fenestrated capillaries may be rather similar. The brain is an obvious exception which will be discussed later. The small intestine may also be an exception having extraction values 3–5 times those in most other tissues. And the lung is another exception. In rabbits the plasma flow through the lung is about 60 ml/min/g (Verner, Bartlett and Johnson 1976). With a turnover rate of 0.58 l/min/g for albumin extraction value is 0.001% which is less than 1/10 of that in most tissues where the blood flow is adjusted to the needs of the tissue.

Approximative values for capillary surface have been reported for some tissues. In rat capillary surface in heart and vasodilated skeletal muscle (Spector 1956) are 800 and 75 cm² respectively. In kidney cortex and medulla in dogs capillary surface areas are 350 cm² and 780 cm²/g respectively (Kügelgren and Braunger 1962). If these values are used for albumin permeability in heart muscle, skeletal muscle, kidney cortex and medulla are 160, 70, 34 and 26 μ l/min/m capillary surface respectively. For IgG the permeability values are 55, 33, 33 and 16 μ l/min/m respectively. Thus the difference in turnover between heart and skeletal muscle is easily accounted for by the difference in capillary surface. And there is no evidence in these data indicating that fenestrated capillaries in general have a markedly higher protein permeability than the nonfenestrated. Hence there is no indication for the presence of more large pores in fenestrated capillaries than in continuous capillaries.

As mentioned in the Introduction no large real pores have ever been observed in continuous capillaries and their presence in fenestrated capillaries is uncertain. In fact, it is only in the small intestine (Simionescu, Simionescu and Palade 1972) that there is some evidence for passage of macromolecules through the fenestrae. Pinocytotic vesicles on the other hand are present both in continuous and fenestrated capillaries and morphologi-

evidence indicates that in both kinds of capillaries they are involved in the transport of macromolecules. The capillaries of the central nervous system represent an interesting exception pinocytosis being scarce and not involved in transport across the endothelial cells. And therefore the capillary permeability to protein is practically zero. One has to consider the possibility when that pinocytosis may account for most of the protein permeability in most fenestrated as well as in most continuous capillaries.

Studies with low molecular weight substances indicate that in skeletal muscle the functional small pores have permeability characteristics similar to those of water-filled pores with diameter of 7-8 nm (see Michel 1972). It seems likely then that in all model 1 tissues there is $P_{\text{H}_2\text{O}}$ and $P_{\text{pinocytosis}}$ and/or filtration through large pores resulting in an outflow of modified plasma and 7-8 nm pores (or equivalent slits) permitting ultrafiltration and diffusion of the low molecular weight substances.

In model 2 tissues albumin—and possibly also IgG—transport through the capillary wall was bidirectional. For two of these tissues, heart muscle and lung, studies with low molecular weight substances have indicated that the small pores have diameters of about 9 nm (see Michel 1972). Such pores would have a certain permeability to albumin (molecular (Stokes-Einstein) diameter 7 nm) but not to IgG (molecular diameter 11 nm). It is possible then that the main difference between model 1 and 2 tissues is that in the latter tissues there is appreciable diffusion of albumin through the small pore system. But diffusion through large pores also may contribute to model 2 behaviour.

Protein dynamics in tissue fluids

As discussed for model 2 B IgG may be more hindered than the albumin on its way to the lymph vessels. The restriction may be located in the tissue or in the walls of the lymph capillaries. In vitro experiments have indicated that the presence in connective tissue of glucosaminoglycans might affect the plasma proteins passing through the tissue. Two types of interference have been discussed (Lauritzen 1970): one is that the tissue fluid has to pass through the gel which may cause filtration effects, the large molecules tending to move at a reduced rate through the gel, another possible interference is that the glucosaminoglycans are present in discrete compartments with the tissue fluid passing in between. Such an arrangement might lead to a gel chromatography effect, the larger molecules passing at higher rates through the tissue than the smaller molecules. In the present experiments IgG had the same or a lower turnover rate constant than the albumin in all tissues permitting comparisons. In the tissues with the same turnover rate constants for the two proteins there was thus no observable interference of the glucosaminoglycans with the protein dynamics. Once outside the capillaries the proteins appeared to pass through the tissue essentially with a bulk flow. Of course this might be a net result—slight gel chromatography effects canceling weak filter effects and effects due to differences in diffusion coefficients.

In the lung with a lower turnover rate constant for IgG than for albumin and a space ratio markedly higher than expected from published lymph data, a combination of model 2 A and B may explain the results. It is not clear if filter effects in the tissue or in the walls of the lymph capillaries or adsorption of IgG to some tissue components was the mechanism causing the large IgG space.

continuous capillaries. And heart muscle is an exception in having continuous capillaries and a high protein permeability. However if extraction values are compared this suggestion becomes highly questionable. Extraction of albumin, E_a , and IgG was defined as the re-uptake rate of the protein in question/regional plasma flow. The outflow of albumin, through a capillary bed into the tissue depends on the permeability of the vessels to albumin, P_a , and the surface of the capillaries, S , and the concentration of albumin in plasma, C_{api} .

$$J = P S C_{api}$$

The amount of albumin entering the capillary bed with the blood depends on the plasma flow Q and the plasma concentration of albumin, C_{api} .

$$\text{Thus } E_a = \frac{P S C_{api}}{Q C_{api}} = \frac{P S}{Q}$$

The conditions for IgG are analogous. The extraction values, Table V in a number of tissues were quite similar irrespective of differences in capillary ultrastructure, Table III. That the plasma flow is related to the number of perfused capillaries and thereby to the capillary surface—which is the case if the average blood flow in the perfused capillaries is about the same in the different tissues—it turns out that the protein permeability of the capillary wall in most tissues with continuous or fenestrated capillaries may be rather similar. The brain is an obvious exception which will be discussed later. The small intestine may also be an exception having extraction values 3–5 times those in most other tissues. And the lung is another exception. In rabbits the plasma flow through the lung is about 60 ml/min/g (Wimers, Bartlett and Johnson 1976). With a turnover rate of 0.38 l/min/g for albumin the extraction value is 0.001% which is less than 1/10 of that in most tissues where the blood flow is adjusted to the needs of the tissue.

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Adsorption of IgG

Anatomical studies have shown that in the liver spleen (see Bennet *et al* 1959), adrenals (Mori and Onoé 1967) and bone marrow (Majno 1966) there are gap capillaries. In the experiments reported here the movement of albumin and IgG out of the blood vessels was so rapid that a turnover rate could not be calculated for any of these tissues. In the liver spleen and adrenals but not in the bone marrow the IgG space was larger than the albumin space in all times. This was a very unexpected result—first observed in preliminary experiments with human IgG. It seemed possible that heterologous or denaturated IgG might be accumulated in the reticuloendothelial cells of the three tissues and for this reason screened rabbit IgG was used. However even then there was accumulation of IgG. Evidence for transient binding of homologous IgG to macrophages obtained from the lung was reported by Phillips-Quagliata *et al* (1971). The present results suggest that there may be a similar phenomenon in the liver spleen and adrenals.

The drainage of tissue fluid

Model 1 as well as 2 A and B assume that there is protein drainage from the tissues by lymph flow. There are two tissues where this does not apply the choroid plexus and the kidney medulla. In these tissues there are no lymph vessels. The routes by which the extravascular proteins leave the choroid plexus are not completely known but drainage into the ventricular fluid is one obvious mechanism that has been considered (Brightman, Reese and Feder 1970). The following calculation suggests that such drainage is the main mechanism. In rabbits the rate of formation of the cerebrospinal fluid is about 10 μ l/min (Welch 1963 Bradbury and Davson 1964). The weight of the choroid plexus in the expts. reported here was about 15 mg. The turnover rate of the albumin corresponded to about 0.9 μ l plasma equivalent fluid per min per gram tissue or 0.014 μ l per min for the whole plexus. If all the albumin were drained into the cerebrospinal fluid the albumin concentration of this fluid would be about 0.14% of that in the plasma. This figure is even less than the mean figure that was reported by Amtorp and Sørensen (1974). In their study the albumin concentration in the plasma was about 40 g/l and that in the aqueous humor about 0.12 g/l. The concentration of albumin in the cerebrospinal fluid thus was about 0.3% of that in the plasma.

In the kidney lymph vessels are present in the cortex but not in the medulla (Kriz and Dieterich 1970). It is paradoxical that the tissue in table 1 having the fastest turnover rate of albumin lacks lymph vessels. At present nothing seems to be known about the drainage of tissue fluid from the medulla. One can only speculate that there may be a sluggish flow of tissue fluid into the lymph vessels of the cortex.

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Influence of Age on Sensitivity and Effector Mechanisms of the Carotid Baroreflex

By

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Abstract

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In 30 healthy subjects aged 20-48 years the hemodynamic response to carotid sinus stimulation (neck tension 40 mmHg) was studied. Heart rate, arterial pressure and cardiac output (dye dilution technique) were measured. In order to evaluate the effect of age on carotid sinus function the material was subdivided into two arbitrary subgroups, aged up to 30 years ($n = 15$) respectively 30 years and above ($n = 15$). Carotid sinus stimulation induced significantly greater reduction in mean arterial pressure in the younger group compared to the older group. The heart rate reduction was, on the average, slightly greater in the younger group though the difference was not significant. In both groups significant decrease in cardiac output contributed to the demonstrated reduction in mean arterial pressure. As the decrease in cardiac output was, on the average, slightly smaller in the younger group, the results indicate that the greater blood pressure response in the younger group was due to greater reduction in peripheral vascular resistance. This is further supported by the findings of significant correlation between changes in total peripheral vascular resistance, elicited by carotid sinus stimulation and age.

Key words: Carotid sinus, pressure receptors, age factors, cardiac output, blood pressure, heart rate, vascular resistance

An inverse relation between age and baroreceptor function has been demonstrated in four recent articles (Bristow and Honour 1969, Eckberg, Drabinsky and Braunwald 1971, Gribble *et al.* 1971 and Duke *et al.* 1976). In these studies the baroreflex was tested by the Valsalva maneuver and/or pharmacologically (angiotensin or phenylephrine) induced blood pressure increase. Quantitatively the baroreflex has usually been evaluated from the decrease in heart rate elicited by the induced increase in blood pressure as suggested by Smyth, Sleight and Pickering (1969). However the primary function of the baroreceptors are to regulate blood pressure. An unimpaired blood pressure regulating capacity of the carotid sinuses can be found during conditions when the effect on heart rate is virtually abolished (Bevegård *et al.* 1977 b). With this background we considered it of interest to study the baroreceptor effect on heart rate and blood pressure separately to further evaluate the effect of age on baroreceptor function.

TABLE 1 Dimensional data. Mean values \pm S.E. I age <30 years, n=15 II age >50 years, n=15 BSA Body surface area from nomogram of Dubois and Dubois (1916).

	Age (years)	Weight (kg)	Height (m)	BSA (m ²)
I	24.8 \pm 0.81	75.7 \pm 2.20	1.79 \pm 0.02	1.95 \pm 0.04
II	36.9 \pm 1.71	78.0 \pm 2.94	1.83 \pm 0.01	2.00 \pm 0.04
Diff	+12.1	+2.3	+0.04	+0.05
Sign of diff	p<0.001	n.s.	n.s.	n.s.

There is disagreement in literature about the extent to which a decrease in cardiac output and/or a reduction in total peripheral vascular resistance contributes to the blood pressure decrease during baroreceptor stimulation (for ref. see Kirschheim 1975). This has also been discussed in some detail in an earlier article (Bevegård, Castenfors and Lindblad 1977c). Therefore we have also tried to elucidate whether age is a factor influencing the relative importance of the two effector mechanisms of the carotid sinus, contributing to the discrepancy stated above. Such data might be of interest since most data in normals refer to young volunteers, whereas data on patients, for example with hypertension or angina pectoris, usually refer to distinctly older subjects.

Material

Data from 30 healthy male volunteers (blood donors) are presented. 20 of the subjects participated in other studies (Bevegård *et al.* 1977 c, d). The material was arbitrarily divided according to age in 2 equal sized groups. Dimensional data for the two groups are given in Table 1.

Methods

Carotid sinus stimulation

The carotid arterial stretch receptors were stimulated by applying subatmospheric pressure (-40 mmHg) round the neck. Details of the stimulation equipment and methods are presented elsewhere (Bevegård *et al.* 1977 b, c).

Haemodynamic measurements

Details concerning catheterization procedure, measurements of mean arterial blood pressure (MAP), heart rate (HR) and cardiac output (\dot{Q}) are given elsewhere (Bevegård *et al.* 1977 a). To minimize differences due to dimensional factors cardiac output, stroke volume and total peripheral vascular resistance (MAP divided by \dot{Q}) are corrected for body surface area (BSA) and presented as cardiac index (CI), stroke index (SVI) and vascular resistance index (TVI) respectively.

Statistical methods

Details are given elsewhere (Bevegård *et al.* 1977 b).

General experimental design

Heart rate, blood pressure and cardiac index were compared during resting condition and during neck suction. The dye injection for determination of cardiac output was made approximately 1 min after the beginning of neck suction. The heart rate presented represents an average during the indicator curve. To obtain comparable and complete data in all subjects only blood pressures immediately prior to cardiac output determination have been used for calculation.

TABLE II Some hemodynamic data during control condition. Mean values \pm S.E. I, age < 30 years, $n = 15$
II, age > 30 years, $n = 15$.

	HR	MAP	CI	SVI	TPI
I	69.3 (± 2.24)	93.7 (± 2.06)	3.5 (± 0.20)	50 (± 2.6)	7.3 (± 0.62)
II	60.7 (± 1.93)	93.3 (± 1.79)	3.3 (± 0.19)	51 (± 2.40)	7.3 (± 0.39)
Difference	8.6	-0.4	0	-1	-0.5
Sign of diff	p 0.01	n.s.			n.s.

Results

Hemodynamic variables during control condition

During control condition (Table II) the younger (I) and the older group (II) showed essentially the same values considering mean arterial pressure, cardiac index, stroke index and total peripheral vascular resistance index whereas the heart rate was slightly higher in the younger group.

Hemodynamic response to stimulation of the carotid sinus

In both groups (Table III) neck suction induced a significant decrease in heart rate, mean arterial pressure and cardiac index, but only in the older group (II) did the reduction in stroke index reach significant level. Total peripheral vascular resistance index decreased significantly in the younger group (I) whereas on the average a minor but not significant increase was found in the older group (II). On comparison of the two groups (Table III) the reduction in mean arterial pressure was significantly greater in the younger group (I), compared to the older group (II). The difference in reaction regarding total peripheral vascular resistance index between the two groups is significant. On the average the heart rate decrease was slightly greater in the younger group whereas the decrease in cardiac index and stroke index were slightly smaller in the younger group though these differences did not reach significant levels.

Changes in total peripheral vascular resistance index in relation to age

On correlating the change in total peripheral vascular resistance index to the age of the subjects (Fig. 1) a significant correlation ($r = -0.61$, $p < 0.001$) is found.

TABLE III Effect of baroreceptor stimulation on heart rate (HR), mean arterial pressure (MAP), cardiac index (CI), stroke index (SVI) and vascular resistance index (TPI). Mean values \pm S.E. I, age < 30 years, $n = 15$; II, age > 30 years, $n = 15$.

Variable	I		II		I-II
	Change during stimulation	Sign. of difference	Change during stimulation	Sign. of difference	Sign. of difference
Δ HR	-3.3 (± 0.73)	$p < 0.001$	-2.1 (± 0.56)	$p < 0.01$	n.s.
Δ MAP	11.5 (± 1.63)	$p < 0.001$	-6.9 (± 1.53)	$p < 0.001$	$p < 0.05$
Δ CI	0.3 (± 0.09)	$p < 0.05$	0.4 (± 0.07)	$p < 0.001$	n.s.
Δ SVI	1 (± 1.07)		-4 (± 1.02)	$p < 0.01$	
Δ TPI	0.4 (± 0.18)	$p < 0.05$	+0.2 (± 0.16)		$p < 0.01$

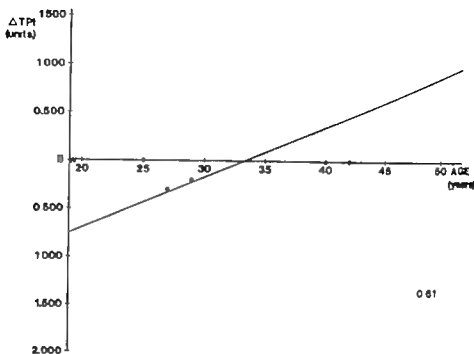


Fig. 1 Correlation between age and change in TPI induced by stimulation of the carotid sinus. TPI = peripheral vascular resistance per square metre body surface area.

Discussion

The function of the arterial baroreceptors has mainly been evaluated by four different methods in man: 1) studying the response to a Valsalva maneuver, 2) studying the cardiac slowing in response to a pharmacologically induced pressure increase, 3) neck suction and electrical stimulation of the carotid sinus. The latter method has, however, been used only in elderly patients (Carlsten *et al* 1958, Tuckman *et al* 1966, Epstein *et al* 1969, Farrell 1972) and thus no correlation with age has been possible.

Studies using the Valsalva maneuver technique (Appenzeller and Descarris 1964, Walther *et al* 1970, Gross 1976) suggest that baroreceptor function decreases with age. However, as emphasized by Duke *et al* (1976) "the Valsalva maneuver test as a measure of intact baroreceptor mechanisms must be interpreted with caution" as it requires the active participation and co-operation of the subject. The Valsalva maneuver can be suspected to be a rather complex stimulus affecting intrathoracic receptors as well as the arterial stretch receptors in the carotid sinus.

Testing the arterial baroreceptors by pharmacologically induced blood pressure increases has been used repeatedly as a quantitative measure to show that baroreceptor function is altered in various situations in man. The method has been evaluated in detail by Smyth *et al* (1969) and Bristow *et al* (1969). With this stimulus a negative relation between age and baroreceptor function has been demonstrated by Bristow *et al* (1969), Eckberg *et al* (1971) and Duke *et al* (1976). Using pharmacologically induced changes in blood pressure, Komor *et al* (1974) demonstrated a diminished baroreceptor heart rate response in older subjects using similar arbitrary subgroups as in this study. However, as emphasized by Duke

et al. (1976) the present test describes baroreceptor function only in terms of cardiac slowing and tells very little about the mechanisms involved. Using this stimulus it is not possible to separate the baroreceptor effect on heart rate and blood pressure.

Neck suction is a stimulus primarily affecting only the carotid sinus, but as emphasized by for example Burton (1965) it is an unphysiological approach as the error signal—an increased blood pressure—is reaching the brain from only one receptor area. Neck suction has been used by several authors (for ref. see Bevegård *et al.* 1977 b) to quantitatively study the carotid buffer reflex. Using this stimulation method, Wagner-Wackerbauer and Hilger (1968) in 26 normal subjects, covering the rather narrow range 23–36 years found no influence of age on the heart rate response of the carotid sinus. The present data demonstrate a greater blood pressure regulating capacity in the younger subjects compared to the older subjects. Considering mean values a similar tendency is found regarding heart rate though the difference did not reach significant level. In this material a correlation, though weak, ($r = 0.4$ $p = 0.05$) is found between changes in mean arterial blood pressure and heart rate induced by neck suction. The present findings thus are in agreement with results earlier demonstrated using the present test.

On dividing the material into arbitrary subgroups according to chronological age, as a substitute for biological age, the individual responses demonstrate a considerable scatter within the two groups. Age is only one of the factors known to influence baroreceptor function. With similar technique as in this study Stegenga, Busert and Brock (1974) have demonstrated reduced baroreceptor function in physically well trained subjects. A dependence of intra-thoracic blood volume on the carotid sinus reflex has also been demonstrated (Bevegård *et al.* 1977 b).

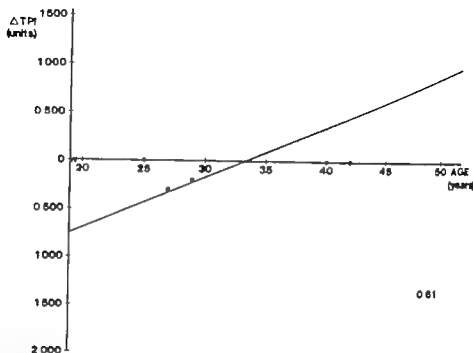
Findings in the literature disagree about the relative importance of the two effector mechanisms of the arterial baroreceptors, changes in cardiac output and vascular resistance.

As summarized in an earlier article (Bevegård *et al.* 1977 c) this lack of agreement applies also to studies in man. The findings in this study of significantly greater reduction in mean arterial pressure in the younger group (I) in spite of an, on the mean, slightly smaller reduction in cardiac index compared to the older group (II), indicate that the reduction in vascular resistance index is greater in the younger group. An age dependent influence on the effect of the resistance vessels elicited by the carotid sinus can also be supported by the finding of a significant correlation between age and the changes in total peripheral vascular resistance index induced by baroreceptor stimulation (Fig. 1).

The effect of age may contribute to the disagreement about the relative importance of the two effector mechanisms stated above. As emphasized by Aars (1975) independent reductions in baroreflex sensitivity with age and hypertension should be taken into account when planning clinical investigations.

Degenerative structural changes with ageing and "stiffening" of the arterial wall have been well documented (Blumenthal, Lansing and Grey 1950, Rouch and Burton 1959, Learoyd and Taylor 1966 and O'Rourke *et al.* 1968).

An increased resistance to deformation could result in less deformation of the arterial wall at the receptor site and hence less baroreceptor stimulus for a given pressure decrease outside the arterial wall. It also seems possible that loss of elasticity in the arterioles results in



0 61

Fig 1 Correlation between age and change in TPI induced by stimulation of the carotid sinus. TPI total peripheral vascular resistance per square metre body surface area.

Discussion

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Studies using the Valsalva maneuver technique (Appenzeller and Descarris 1964, Wade *et al* 1970, Gross 1976) suggest that baroreceptor function decreases with age. However as emphasized by Duke *et al* 1976 "the Valsalva maneuver test as a measure of intact baroreceptor mechanisms must be interpreted with caution" as it requires the active participation and co-operation of the subject. The Valsalva maneuver can be suspected to be a rather complex stimulus affecting intrathoracic receptors as well as the arterial stretch receptors in the carotid sinus.

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in a lesser change in vascular tone in response to the same change in neurogenic constrictor tone. Also an influence of age on the central nervous control of the baroreceptor system and/or peripheral nervous system might contribute to the findings.

In conclusion the results indicate that the carotid sinus induced hemodynamic changes are influenced by the age of the subject. This seems to be true considering both the magnitude of blood pressure response as well as considering the relative importance of the two effect mechanisms—reductions in cardiac output and total peripheral vascular resistance.

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These findings motivated the present study to evaluate the relative importance of the two blood pressure reducing mechanisms on stimulation of the carotid sinus—reduction in cardiac output and reduction in total peripheral vascular resistance—under conditions with normal and reduced central blood volume.

Material and methods

Subjects. 10 healthy male volunteers (blood donors) participated. Mean values \pm S.D. are for age 29.6 ± 3.9 years, for height 1.82 ± 0.06 m and for weight 80.4 ± 9.3 kg. A separate study of the heart rate response was made in six healthy subjects, mean values \pm S.D. are for age 30.0 ± 6.5 years, for height 1.83 ± 0.04 m and for body weight 72.2 ± 6.9 kg.

Carotid sinus stimulation. The carotid arterial stretch receptors are stimulated by airtight the pressure in a plexiglass box enclosing the neck air-tightly. The pressure is lowered rapidly from atmospheric to -40 mmHg and kept constant during the stimulation period. Details of the stimulation equipment are presented elsewhere (Bevergård *et al.* 1977b).

Lower body negative pressure. The lower body (below the iliac crest) is enclosed in an air-tight box. With the aid of a high capacity electric pump, the pressure in the box could be lowered rapidly to -40 mmHg below ambient and kept constant during the stimulation period. The procedure will be referred to as lower body negative pressure (LBNP -40). For technical details see Bevergård, Castenfeldt and Lindblad (1977).

Catheterization procedures and methods. Details concerning statistical methods, catheterization procedures, measurements of mean arterial pressure (MAP), central venous pressure (CVP), heart rate (HR), cardiac output (\dot{Q}) and forearm blood flow (FBF) are given elsewhere (Bevergård *et al.* 1977). Total peripheral vascular resistance (TPVR) and regional vascular resistance (RVR) are calculated as Δ MAP divided by \dot{Q} respectively forearm blood flow (FBF) and expressed in arbitrary units.

General experimental design. The subjects participated in an initial twelve-minute experimental run focusing on the dynamic features of the carotid baroreflex during gradually decreasing levels of LBNP. These data are presented in a separate article (Bevergård *et al.* 1977b). After re-gaining stable conditions, estimated from heart rate and blood pressure, the experimental scheme summarized in Fig. 1 was commenced. Cardiac output (\dot{Q}) and forearm blood flow (FBF) determinations were made as indicated by the heavy lines (Fig. 1). The heart rate (HR) presented under benefits represents the mean value for the period between cardiac output as determined. Intravascular pressures were measured in the intervals between \dot{Q} determinations. The values presented are an average of measurements immediately before and after the determination of \dot{Q} .

In 10 subjects \dot{Q} was determined not only before and during but also after carotid sinus stimulation during continuous LBNP. In these 10 subjects the LBNP period was extended approximately 5 min.

In 6 subjects a separate study was made of the heart rate response to the same baroreceptor stimulus under control conditions, LBNP -40 and 60° head up tilt. T illustrates the time sequence of the heart rate response the heart rate is averaged in fifteen second periods.

Results

Circulatory effects of LBNP -40. Table I gives the values collected at \dot{Q}_1 and \dot{Q}_2 according to Fig. 1. Compared with control conditions, LBNP -40 induced a significant decrease in central venous pressure, whereas mean arterial pressure was essentially unchanged. Heart rate increased 5.2 beats/min which was not enough to compensate for a decrease of stroke volume by 26 ml, thus \dot{Q} on the average decreased 1.3 l min^{-1} . Total peripheral vascular resistance and regional vascular resistance both increased significantly.

Hemodynamic response to stimulation of the carotid sinus

Control conditions. Table II A presents the differences between the values collected at \dot{Q}_1 and \dot{Q}_2 according to Fig. 1). Neck suction induced a significant reduction in mean arterial

Effect of Carotid Sinus Stimulation on Cardiac Output and Peripheral Vascular Resistance during Changes in Blood Volume Distribution in Man

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Abstract

BEVEGÅRD S., J. CASTENFORS and L. E. LINDBLAD. *Effect of carotid sinus stimulation on cardiac output and peripheral vascular resistance during changes in blood volume distribution in man*. Acta physiol. scand. 1977 101 50-57

In ten healthy subjects (mean age 29.6 years) the hemodynamic response to carotid sinus stimulation (neck suction -40 mmHg) was studied under control conditions and during peripheral pooling of blood (lower body negative pressure). Heart rate, arterial and central venous pressure, cardiac output and forearm blood flow were measured. The time sequence of the heart rate response was studied separately in six beds in the subjects. During control conditions, carotid sinus stimulation induced a significant decrease in arterial pressure and heart rate. The blood pressure decrease mainly reflected reduction in cardiac output, with peripheral vascular resistance being essentially unchanged. However, in the skeletal muscle, represented by a forearm segment, vascular resistance decreased significantly. During lower body negative pressure (LBNP) the same stimulation of the carotid sinus induced a significantly greater fall in mean arterial pressure even though the reduction in cardiac output was slightly smaller on the average than in the control condition. The heart rate increased probably secondary to time dependent increases in heart rate elicited by the carotid sinus stimulus. Total peripheral vascular resistance decreased significantly during LBNP, the reaction likewise differing significantly from that in the control condition. Thus the appropriate blood pressure response was due to a more pronounced vasodilatation when the carotid sinus was stimulated during lower body negative pressure. The results indicate that the hemodynamic changes elicited by carotid sinus stimulation are modified by changes in the distribution of blood volume and in the type of resistance vessels.

Key words. Carotid sinus, blood volume, central venous pressure, pressure receptors, cardiac output, blood pressure, vascular resistance

Previous studies (Bevegård *et al.* 1977 b) have demonstrated that the effect of a standardized baroreceptor stimulus (sinusoidal changes in the pressure around the neck) on heart rate and blood pressure is modified by changes in blood volume distribution, possibly via stimulation of intrathoracic low pressure receptors. Thus, during lower body negative pressure (-40 mmHg) the blood pressure regulating capacity of the carotid sinus is augmented while the heart rate response is distorted and difficult to interpret.

TABLE II. Hemodynamic response to baroreceptor stimulation under control conditions (A) and lower body negative pressure (LBNP) (B) ($n=10$). Mean values \pm S.E.

	A		B		A-B
mean arterial pressure (mmHg)	-8.5 ± 1.7	p 0.01	-13.3 ± 1.2	p<0.001	p<0.01
cardiac output ($l \cdot min^{-1}$)	-0.7 ± 0.2	p 0.01	-0.4 ± 0.1	p<0.05	s
heart rate (beats min^{-1})	3.0 ± 1.0	p 0.05	+3.2 ± 0.8	p<0.001	p<0.001
stroke volume (ml)	-8.1 ± 2.4	p<0.05	11.7 ± 1.9	p<0.001	s
total peripheral vascular resistance (mmHg)	+0.1 ± 0.4	s	1.2 ± 0.4	p<0.05	p<0.01
brachial blood flow (ml $min^{-1} 100 ml^{-1}$)	+0.2 ± 0.2	s	0.0 ± 0.3	s	
leg/arm vascular resistance (mmHg)	7.1 ± 2.5	p<0.05	-7.9 ± 3.6	p 0.05	s

in cardiac output during LBNP compared with control conditions, $-0.4 l \cdot min^{-1}$ and $-0.7 l \cdot min^{-1}$ respectively (the difference not being significant). In two cases, as described, the LBNP period was extended so that cardiac output could also be determined after the end of neck suction. In both subjects cardiac output increased after the end of neck suction, in spite of persistent LBNP stimulation (Fig. 2).

The heart rate response also differed significantly between the two conditions, changing from a decrease in the control period to an increase during LBNP. The time sequence of the heart rate response during LBNP (Fig. 3) suggests that this is due to a gradual time-dependent decrease in heart rate, interrupted by a transient decrease in heart rate shortly after the application of neck suction.

Time sequence of heart rate response to neck suction under control conditions, LBNP 40 and head-up tilt (60°) (Fig. 4)

Under control conditions there was an immediate significant decrease in heart rate which sustained during the stimulation period. During LBNP and head-up tilt (60°) there was a less marked and also a less well sustained decrease in heart rate, especially in the tilt condition.

Neck suction
(mm Hg)

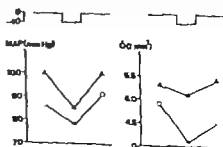


Fig. 2. Mean arterial pressure (MAP) and cardiac output (CO) in two subjects before, during and after neck suction and intermittent lower body negative pressure (LBNP).

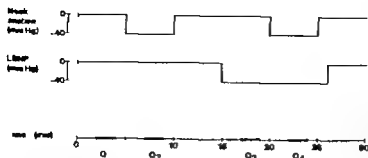


Fig. 1 General experimental design. Heavy lines indicate the time when cardiac output and forearm blood flow was measured. (LBNP=lower body negative pressure)

pressure as well as in heart rate, cardiac output and stroke volume. Total peripheral vascular resistance was essentially unchanged, whereas regional vascular resistance in the forearm decreased significantly.

LBNP -40 (Table II B presents the differences between values collected at \dot{Q} and \dot{Q} according to Fig. 1). Also during LBNP, neck suction induced a significant decrease in mean arterial pressure, cardiac output and stroke volume, whereas heart rate increased significantly. Total peripheral vascular resistance and regional vascular resistance both decreased significantly.

Hemodynamic response of neck suction during LBNP compared to control conditions (Table II A-B)

Compared with control conditions, neck suction elicited a significantly greater reduction in mean arterial pressure during LBNP. The significant decrease in total peripheral vascular resistance during LBNP differed significantly from the reaction found during control conditions, whereas regional vascular resistance in the forearm showed essentially the same significant decrease under both conditions. On the average there was a less marked decrease

TABLE I. Effect of lower body negative pressure -40 mmHg (LBNP -40) on some hemodynamic variables (n=10). Mean values \pm S.E.

	Control	LBNP -40	Diff.	Sign. of diff.
Mean arterial pressure (mmHg)	95.5 ± 1.8	96.8 ± 1.6	+1.3 ± 0.9	n.s.
Central venous pressure (mmHg)	7.9 ± 0.6	3.4 ± 0.6	-4.4 ± 0.3	$p < 0.001$
Cardiac output (l min ⁻¹)	6.6 ± 0.4	3.4 ± 0.3	-3.3 ± 0.2	$p < 0.001$
Heart rate (beats min ⁻¹)	64.0 ± 2.7	69.2 ± 3.3	+5.2 ± 1.9	$p < 0.05$
Stroke volume (ml)	106 ± 4	79 ± 3	-26 ± 3	$p < 0.001$
Total peripheral vascular resistance (units)	14.7 ± 0.7	18.3 ± 0.8	+3.6 ± 0.4	$p < 0.001$
Forearm blood flow (ml min ⁻¹ 100 ml ⁻¹)	2.9 ± 0.4	1.9 ± 0.2	-1.0 ± 0.4	$p < 0.01$
Regional vascular resistance (units)	36.8 ± 3.5	54.7 ± 4.9	+17.9 ± 2.2	$p < 0.001$

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Mean arterial pressure (mmHg)	-8.3 ± 1.7	$p < 0.01$	-13.3 ± 1.2	$p < 0.001$	$p < 0.01$
Cardiac output ($l \text{ min}^{-1}$)	-0.7 ± 0.2	$p < 0.01$	-0.4 ± 0.1	$p < 0.05$	\pm
Heart rate (beats min^{-1})	-3.0 ± 1.0	$p < 0.05$	+3.2 ± 0.8	$p < 0.001$	$p < 0.001$
Stroke volume (ml)	-8.1 ± 2.4	$p < 0.05$	-11.7 ± 1.9	$p < 0.001$	\pm
Total peripheral vascular resistance (cgs)	+0.1 ± 0.4	\pm	-1.2 ± 0.4	$p < 0.05$	$p < 0.01$
Forearm blood flow (ml cm^{-1} (100 ml^{-1})	0.2 ± 0.3	\pm	0.0 ± 0.3	\pm	\pm
Regional vascular resistance (cgs)	7.1 ± 2.5	$p < 0.05$	-7.9 ± 2.6	$p < 0.05$	n.s.

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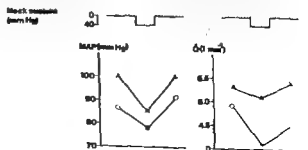


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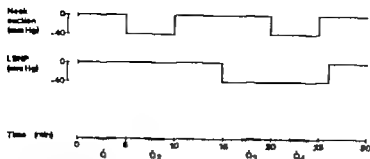


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Regional vascular resistance (units)	36.8 ± 3.5	34.7 ± 4.9	+17.9 ± 2.2	$p < 0.001$

—n cardiac output contributes to the blood pressure reduction on stimulation of the carotid sinus (for ref. see Kirchheim 1976). This lack of agreement applies even to studies in man.

—Using electrical stimulation of the carotid sinus nerve, Carlsten *et al.* (1958) reported that patients under general anaesthesia displayed bradycardia and a decreased pulse pressure, suggesting a reduction in cardiac output. In conscious subjects using a similar stimulation technique, moderate decreases in cardiac output were demonstrated by Tuckman *et al.* (1966) in subjects with severe hypertension and by Epstein *et al.* (1969) and Farrehi (1972) in subjects with coronary artery disease. However their results are difficult to compare with findings in healthy subjects. In six healthy subjects Bevegård and Shepherd (1966) found that cardiac output fell 0.6 l min^{-1} when the pressure around the neck was reduced 40 mmHg. In twenty-three hypertensive subjects (mean age 43 years), Castenfors, Bevegård and Danielsson (1976) reported that the same stimulus elicited a significant reduction in cardiac output (0.7 l min^{-1}).

On the other hand, Ernesting and Perry (1957), using the same stimulus in two subjects, found no significant difference in cardiac output. Recently Bjurstedt *et al.* (1975) using a similar stimulus (subatmospheric pressure 40 mmHg in a plexiglass helmet enclosing the whole head) in eight healthy subjects reported a slight average increase (0.3 l min^{-1}), though the change was not significant. The reduction in mean arterial pressure was due to significant decrease in total peripheral vascular resistance. This discrepancy may be due, at least in part, to the fact that their subjects were younger (mean age 24.8 years) than those in the present study. A separate presentation of the influence of age on sensitivity and effector mechanisms of the carotid baroreflex in the extended age range 20–50 years reveals an age dependence of the relative importance of reductions in cardiac output and total peripheral vascular resistance respectively (Lindblad, 1977). A decrease in total peripheral vascular resistance is thus mainly found in younger subjects.

In a separate study (Bevegård *et al.* 1977 b) using sinusoidally varying pressure around the neck to obtain standardized carotid sinus stimulus suitable for harmonic analysis, the results indicated an augmented blood pressure regulating capacity during LBNP. The present study confirms this finding.

The present data show the expected heart rate decrease during control conditions but an increase in heart rate during LBNP; this increase is probably at least partly related to the LBNP stimulus per se, since heart rate increased during the continuous LBNP stimulus (Fig. 3). When studying the heart rate response to neck suction during orthostatic stimulation, accomplished through head-up tilt or simulated through LBNP, it is necessary to allow for a base line drift (Fig. 3–Fig. 4). Taking this drift into account, the baroreceptor stimulation seems to have induced a small and non-significant decrease in heart rate during LBNP as well as during tilt. This agrees with the findings during LBNP in the study mentioned earlier using sinusoidal variation of the pressure around the neck (Bevegård *et al.* 1977 b). A decreased vagal tone during LBNP might be of importance for the reduced heart rate response (Eckberg, Fletcher and Braesswald 1972). The reduction in cardiac output was smaller during LBNP than during control conditions. To verify that the small reduction in cardiac output on neck suction during LBNP was due to neck suction and did not just represent the time-dependent gradual decrease during the continuous LBNP stimulus, cardiac output was

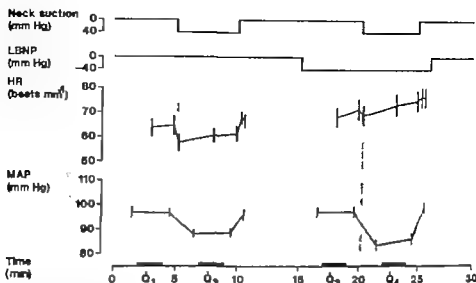


Fig. 3 Time sequence of the heart rate and blood pressure response to neck suction under control conditions and lower body negative pressure (LBNP). Mean values \pm S.E. Sufficient data for this plot are available only eight of the subjects.

Discussion

The hemodynamic effects of LBNP -40 have been discussed in a separate study (Bevegård *et al* 1977a) where the results were similar to those found in the present material. As discussed in the earlier report, it is probable that reflexes originating in the low pressure area at least contribute to the demonstrated vasoconstriction, resulting in the essentially unchanged mean arterial pressure in spite of the demonstrated reduction in cardiac output.

The reflexly induced decrease in heart rate and reduction in blood pressure during carotid stimulation by neck suction are well known (Ernsting and Parry 1957, Bevegård and Shepherd 1966, Thron *et al* 1967, Wagner, Wachsbauer and Hilger 1968, Beiser *et al* 1969, Stegmann, Busert and Brock 1974, Bjurstedt, Rosenhamer and Tydén 1975, Abboud *et al* 1975).

In this material baroreceptor stimulation during control conditions resulted in a significant reduction in cardiac output by 0.7 l min⁻¹ and essentially unchanged total peripheral vascular resistance. Findings in the literature disagree about the extent to which a decrease

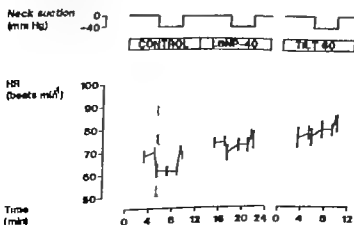


Fig. 4 Time sequence of heart rate response to neck suction during control conditions, lower body negative pressure -40 mmHg (LBNP -40) and head-up tilt (-6°). Mean values \pm S.E.

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determined in two cases before, during and directly after neck suction (Fig. 2). In these two subjects, no indication of a gradual time-dependent drop in cardiac output was found during the period studied.

The finding that LBNP stimulation augmented the blood pressure reduction during neck suction in spite of a smaller reduction in cardiac output indicates that the decrease in peripheral vascular resistance is greater under this condition. This is supported by the finding that the reduction in total peripheral vascular resistance during neck suction was significant only during LBNP.

In the skeletal muscle (represented by a forearm segment) the finding of an unchanged forearm blood flow during neck suction in spite of a reduced mean arterial pressure suggests a dilatation of the resistance vessels under resting conditions (Bevegård and Shepherd 1966, Beiser *et al.* 1969). In this study similar results were found during both control and LBNP conditions.

In conclusion, the results suggest that both a reduction in cardiac output and a decrease in peripheral vascular resistance may contribute to the carotid sinus induced reduction in blood pressure but the relative importance of the two effector mechanisms depends on the hemodynamic situation. The hemodynamic adjustments induced by LBNP result in a decrease in blood pressure but a decrease in the heart rate regulating capacity of the carotid sinus.

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Methods

male rats of Sprague-Dawley strain bred at this Institute and weighing about 350 g at the start of the experiment are used. Under ether anesthesia one of the following three surgical procedures as performed: section of the hypogastric nerve on both sides distal to the hypogastric ganglia; below the bifurcation of the aorta section of the pelvic nerves close to the urinary bladder and extirpation of the celiac ganglion on both sides; and combination of these two procedures. After the two latter types of operation, the bladders of the rats had to be emptied daily by manual pressure using ether anesthesia. And bilateral section the animals were given salformamide (Solform[®] Nordmark Werke), 20 mg daily subcutaneously 3 or 8 days after the operation the rats were either killed by exsanguination or the bladders removed for choline acetyltransferase assay or they were anesthetized with chloralose (100 mg/kg) through femoral cannula after sedation with ether and used for experiments in which the sympathetic nerves were stimulated electrically.

Assay of choline acetyltransferase

The bladders of 36 rats were analysed; unoperated litter mates served as controls. The method used is that devised by Catherine Fiebb (see Nordmark 1963). Acetone dried powder of the bladders was prepared and then made up as cysteine-saline in concentration of 25 mg/ml or less the enzyme activity was expected to be low as in the case after extirpation of the pelvic ganglia alone or in combination with section of the hypogastric nerves, in concentration of 50 mg/ml. Of the tissue extracts 0.1 ml were incubated at 38°C for 1 h. After the incubation had stopped the incubate volumes were adjusted to 40 ml of frog Ringer's solution or less low enzyme activity was expected to 20 ml. The acetylcholine synthesized was then assayed on the electrified frog rectus muscle. When gallamine (4 $\cdot 10^{-4}$ g/ml) was added to the bath the muscle caused no contraction of the muscle and when the incubate was boiled for short time in water (but not in acid) its activity was destroyed, both findings, both seem to confirm that the active substance studied is acetylcholine.

The enzyme activity is expressed as μ g acetylcholine chloride formed per h per urinary bladder (total activity) and as μ g acetylcholine chloride formed per h per g acetone powder (concentration). The incubation medium was such that the activity of rabbit brain, used as reference sample, was 9 200 μ g ACh/h/g acetone powder. Activities less than 10 μ g ACh/h/g acetone powder could not be measured on the rectus muscle.

Stimulation experiments

All rats were used. The bladder was exposed and the ureters are ligated. A glass cannula is inserted into the bladder through an incision in the urethra. The bladder was filled with 0.25 ml of physiological saline and the pressure development by the detrusor muscle was recorded by means of transducer and polygraph. The mean pressure is about 5 mmHg in all experiments.

The hypogastric nerves were cut distal to the hypogastric ganglia and the distal ends were stimulated electrically at about the level of the bifurcation of the aorta, using bipolar electrode. A Grass stimulator, giving rectangular pulses with duration of 2 ms, frequency of 20 or 100 Hz and of supermaximal intensity (10 V) was used.

Statistics

Student's *t*-test for paired samples was used; comparisons are made between the operated rat and its unoperated litter mate. The 0.05 level of probability is accepted as significant.

Drugs

The substances used in the stimulation experiments are: hexamethonium bromide, streptozalate, dihydroergotamine mesylate and guanethidine sulphate. The drugs were injected through the cannula in the femoral vein.

Results

Activity of choline acetyltransferase

The total enzyme activity of the normally innervated urinary bladders in the 20 rats studied, expressed in μ g acetylcholine formed per h per bladder was 10.2 ± 0.7 (mean \pm S.E.). The

Choline Acetyltransferase Activity in the Denervated Urinary Bladder of the Rat

By

JÖRGEN EKSTRÖM and MATS ELMÉR

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Abstract

EKSTRÖM J and M ELMÉR *Choline acetyltransferase activity in the denervated urinary bladder of the rat* Acta physiol. scand. 1977 101 58-62.

After extirpation of the pelvic ganglia the choline acetyltransferase activity decreased markedly indicating that most of the postganglionic cholinergic neurones of the bladder take this route. A small decrease in the activity of this enzyme was found after section of the hypogastric nerves, showing that these nerves contribute to some extent to the cholinergic innervation. The residual enzyme activity found after a combination of the two surgical procedures suggests that neurones relay distal to the level of the section of the hypogastric nerves and pass outside the pelvic ganglia and (or) that neurones pass the pelvic ganglia and relay distal to them. Electrical stimulation of the hypogastric nerves after extirpation of the pelvic ganglia and the use of blocking drugs showed the existence of cholinergic neurones passing outside the pelvic ganglia, some of them relaying distal to the point of stimulation.

Key words: Urinary bladder choline acetyltransferase, denervation

Stimulation experiments have shown that the detrusor muscle of the rat receives cholinergic fibres not only from the pelvic but also from the hypogastric nerves (Elmér 1975 a). The number of acetylcholinesterase positive nerves in the bladder however does not seem to change by section of the hypogastric nerves, while no such nerves could be found after extirpation of the pelvic ganglia (Alm and Elmér 1975) the hypogastric nerves are considered to pass through the pelvic ganglia (Langworthy 1963). As to the acetylcholine content in the rat bladder about 10 per cent is still found after extirpation of both pelvic ganglia (Carpenter and Rubin 1967). However motor responses could not be elicited by transurethral stimulation of bladders thus operated (Carpenter and Rand 1965).

In this connection it appeared to be of particular interest to study the effects of section of the hypogastric nerves or extirpation of the pelvic ganglia, or a combination of the two procedures on the activity of the acetylcholine forming enzyme, choline acetyltransferase, previously shown to be present in the rat bladder (Ekström 1975 a). The results obtained led to experiments in which the motor effects of electrical stimulation of the hypogastric nerves after extirpation of the pelvic ganglia were studied.

stimulation experiments

Electrical stimulation of the hypogastric nerves at 20 Hz 8 days after extirpation of the pelvic ganglia caused contraction of the detrusor muscle in 8 out of 10 rats. The intravesical response was 1.7 ± 0.4 mmHg ($n=8$). After the injection of hexamethonium, 10 mg/kg, the contraction of the bladder was reduced in 2 out of 4 rats, the reduction being about 50%. Atropine, 1 mg/kg, reduced the response by about 60% in all the rats, while dihydroergotamine, 2 mg/kg, had no effect (Fig. 1).

Stimulation of the hypogastric nerves at 100 Hz gave a response in all the rats and increased the intravesical pressure by 2.0 ± 0.4 mmHg ($n=10$), which was significantly higher than response to stimulation at 20 Hz ($p < 0.05$). The increased part of the response caused by the high stimulation frequency was not affected by previous injection of hexamethonium, 10 mg/kg, or atropine, 1 mg/kg, but was totally abolished by guanethidine, 1 mg/kg, or dihydroergotamine, 2 mg/kg (Fig. 1).

Discussion

The profound fall in the total activity of the choline acetyltransferase in the bladder to about 1 per cent or less of the normal value after bilateral extirpation of the pelvic ganglion, shows that most of the cholinergic postganglionic neurones reach the urinary bladder via this pathway. The decrease in total enzyme activity observed at an early stage after severance of the hypogastric nerves shows that these nerves contribute to the choline acetyltransferase activity of the bladder and thus, supply the bladder with cholinergic neurones. The finding of a residual enzyme activity when extirpation of the pelvic ganglia was combined with section of the hypogastric nerves suggests the presence of cholinergic neurones relaying distal to the level where the hypogastric nerves were sectioned and passing outside the pelvic ganglia and (or) of cholinergic neurones passing the pelvic ganglia and relaying distal to them. The possibility that some fibres of the hypogastric nerves actually pass outside the pelvic ganglia was confirmed by the increase in intravesical pressure when the hypogastric nerves were stimulated after the pelvic ganglia had been extirpated in advance. That the hypogastric fibres stimulated at physiological frequency were cholinergic is suggested by the outcome of the experiments where atropine and dihydroergotamine were used. From this type of experiment, using the ganglion blocking substance hexamethonium, it appears also that ganglion cells may occur distal to the stimulation point. It should be pointed out that the partial resistance to atropine observed is a well known finding (see Elmér 1975 b, Carpenter 1977). High stimulation frequencies are known to stimulate the adrenergic fibres of the hypogastric nerves activating excitatory α -adrenoceptors in the rat bladder (Elmér 1957 c). By the use of such a high frequency in the present study it is shown that also adrenergic fibres reach the bladder without passing the pelvic ganglia, however they apparently do not relay below the level of stimulation. Concerning the possible presence of ganglion cells distal to the anatomically distinct pelvic ganglion Alm and Elmér (1975) stimulated the nerve close to the bladder but the effects did not appear to be blocked by hexamethonium. A small number of intramural ganglion cells, thought to be cholinergic, has been found in some morphological studies on the rat bladder (El-Badawi and Schenk 1966, Chamber 1967).

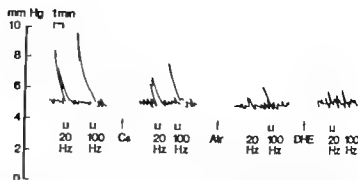


Fig. 1 Pressure responses of a urinary bladder to electrical stimulation of the hypogastric nerve extirpation of the pelvic ganglia. Ca hexamethonium, 10 mg/kg, Atr atropine, 1 mg/kg, DHE, ergotamine, 2 mg/kg.

concentration of the enzyme activity in μg acetylcholine formed per h per g acetone powder was 837 ± 56 . The bladders weighed 73.1 ± 1.9 mg.

The enzyme activity was markedly lower in the bladders of the rats from which the ganglion had been extirpated bilaterally 8 days previously than in the normal controls. In 2 of the 8 bladders examined it was, in fact, below the limit of detection (less than 0.31 μg ACh/h/bladder). In the remaining 6 the total enzyme activity and the concentration (per g acetone powder) were 0.51 ± 0.07 and 15 ± 2.2 μg ACh, respectively. The bladders after this type of operation were found to be 3–4 times heavier than those supplied with normal innervation.

In the 10 rats in which the hypogastric nerve had been sectioned bilaterally 8 days previously the total enzyme activity of the bladders was almost significantly higher ($p < 0.05$) than that of the controls, being 9.6 ± 1.3 and 7.8 ± 1.0 μg ACh, respectively. In terms of concentration (per g acetone powder) the activity was 850 ± 102 and 691 ± 72 μg ACh. This difference which is significant at the $p < 0.01$ level. The weights of the bladders of the two groups did not differ from each other.

In contrast to the above mentioned finding at 8 days urinary bladders of 10 rats examined for their choline acetyltransferase activity 3 days after the section of the hypogastric nerve had a total activity which was 16% lower ($p < 0.01$) than in the 10 control rats, being 10.4 ± 0.5 and 12.5 ± 0.5 μg ACh, respectively. The concentration (per g acetone powder) of the enzyme activity in these operated rats was 884 ± 46 and in the control rats 982 ± 59 μg ACh. This difference tended to be significant ($0.05 < p < 0.1$). When examined at this early stage the bladders of the operated rats were found to be 9% lighter ($p < 0.05$) than those of the respective controls.

Extirpation of the pelvic ganglia combined with bilateral section of the hypogastric nerve did not further reduce the very low acetylcholine forming capacity seen after the section of the ganglia alone. Thus 8 days after the combined surgical procedure 1 out of 10 bladders studied had an enzyme activity which was not detectable (less than 0.33 μg ACh/h/bladder) while the total enzyme activity in the others was 0.82 ± 0.17 μg ACh. In terms of concentration (per g acetone powder) the activity was 23 ± 4.8 μg ACh. The bladders showed a similar weight increase as that seen after extirpation of the pelvic ganglia alone.

Adenosine in Rat Cerebral Cortex. Its Determination, Normal Values, and Correlation to AMP and Cyclic AMP during Shortlasting Ischemia

By

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Abstract

NORDSTRÖM, C. H., S. REHNCRONA, B. K. SÖRBO and E. WESTERBERG. *Adenosine in rat cerebral cortex. Its determination, normal values, and correlation to AMP and cyclic AMP during shortlasting ischemia.* Acta physiol. scand. 1977 101 63-71

has recently been suggested that adenosine is a metabolic coupling factor responsible for an increased regional blood flow during hypoxia or increased functional activity. However, these adenosine concentrations have been reported to increase in situations previously shown to be associated with changes in the AMP concentrations. The present experiments were undertaken to assess cerebral cortex concentrations of adenosine under normal circumstances, and to relate changes in adenosine, AMP and cyclic AMP during shortlasting ischemia. Following freezing and extraction of tissue, adenosine was measured using high pressure liquid chromatography. In paralyzed and anesthetized (70% N_2O) rats, freezing of three brain areas still gave an adenosine concentration of $0.9 \pm 0.1 \mu\text{mol kg}^{-1}$ (mean \pm S.E.M.). With freezing through the exposed dura the concentration was 3 times as high with large scatter. When special measures were taken to avoid tissue trauma during translocation the adenosine concentration was $1.1 \pm 0.1 \mu\text{mol kg}^{-1}$. It is concluded that previously reported values are erroneously high. During the last 60 s of total ischemia there is a linear correlation between increase in AMP and in adenosine concentrations as well as between adenosine and cyclic AMP concentrations. It is concluded that increases in these adenosine concentrations only occur if AMP accumulates. However, since (relative) changes in adenosine concentrations are at least twice those of AMP, analyses of adenosine may provide sensitive measures of change in phosphorylation state.

Current interest in adenosine in the brain centers on two aspects of its metabolism, related to regulation of neuronal function and cerebral blood flow respectively. First, adenosine is postulated to activate adenylate cyclase and thereby to cause increased formation of cyclic AMP (Sartin and Rall 1970, McIlwain 1972). Second, when locally applied to cerebral cortex adenosine has been shown to be a potent vasodilator (Berne *et al.* 1974, Wahl and Kuchinsky 1976). As a result of these and other observations it has been suggested that adenosine acts as one of the regulators of cerebral blood flow during physiological conditions (Rubio and Berne 1975, Rubio *et al.* 1975).

In other studies no ganglia could be found in the bladder wall of this species (Carpenter Rubin 1967 Alm and Elmér 1975)

The observation that the choline acetyltransferase activity was not decreased, but intended to be increased, in the urinary bladder after cutting the hypogastric nerves previously was surprising. This phenomenon may be due to an increase of the activity of the enzyme in the cholinergic nerves present and (or) to an extension of these nerves in the bladder both possibilities that could be explained by an increased nervous activity as a response to the loss of one of the nervous pathways. Other experiments mainly on salivary glands indicate that the activity of choline acetyltransferase is dependent on the traffic of impulses (see Ekström 1975 b, 1977 a, b). It should be pointed out, that the effect of the enzyme activity is probably not a consequence of the degeneration of the adrenergic nerves of the hypogastric nerves, since treatment with 6-hydroxydopamine causing a selective destruction of neurones of this type is not followed by any change in the choline acetyltransferase activity of the urinary bladder of the rat (Ekström 1975 a).

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contraction due to slight tissue trauma, the results do not lend themselves to temporal analysis of changes in adenosine concentration during ischemia. However the results allowed correlation between the concentrations of adenosine, AMP and cyclic AMP.

All brains were chilled out during intermittent irrigation with liquid nitrogen and stored at -80°C until extraction.

Whole brain extracts. The cerebral cortex below the craniotomy and the corresponding area in the non-operated animals are dissected at -22°C in a refrigerated glove box. The samples were weighed and homogenized with HCl-methanol. The tubes were then brought to 0°C , perchloric acid was added and the fraction was completed as previously described (Folbergrova *et al.* 1972). Phosphocreatine (PCr), ATP, DP, AMP, lactate and pyruvate were measured with enzymatic fluorometric methods (Lowry and Passmore 1972, see Folbergrova *et al.* 1972). Cyclic AMP was determined according to Gilman (1970), as described elsewhere (Folbergrova *et al.* 1977).

Analysis of adenosine. were performed with high pressure liquid chromatography (Waters, model U6K universal injector, 6000 A Solvent Delivery System, 450 UV Absorbance Detector). A nonpolar column Bondapak C18 as used as the stationary phase and mixture of 90 ammonium phosphate buffer and 10 methanol, pH 7.1 as the mobile phase (reversed phase operating mode). The absorbance was measured at 254 nm. One hundred μl of the neutralized brain extract was injected directly into the column. Adenosine showed retention time of about 12 min (see below), with an eluent flow of 1.0 ml min^{-1} . The detector sensitivity was 0.0025 AUFS (Absorbance Units Full Scale). Adenosine was identified by comparison of absorbance maxima for variety of nucleotides, nucleosides and bases (see Results).

Adenosine. The energy charge of the adenosine nucleotide pool (E.C.) is calculated according to Atkinson (1968) as

$$\text{E.C.} = \frac{[\text{ATP}] + 0.5 [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

Statistical differences are evaluated with the student's *t*-test and linear regression analyses according to conventional methods.

Materials. Bondapak C18 column (27314) was obtained from Waters Associates, Inc., Gotohburg, MA. Methanol Pronalys was obtained from May & Baker Ltd Dagenham, England, and ammonium phosphate for buffer solutions from Merck Darmstadt, West Germany. Adenosine was supplied by Boehringer Mannheim, West Germany. Cyclic AMP assay kit (TRK-432) from The Radiochemical Centre, Amersham, England, was used for ^3H -AMP analyses.

Results

Measurements of adenosine concentration

The Waters model 440 UV detector is designed for a recorder input of 10 mV giving a minimal sensitivity of 0.005 AUFS. The sensitivity was increased to 0.0025 AUFS by reducing the input potential to 5 mV. A further reduction of input potential resulted in a substantial baseline noise.

Fig. 1 gives the retention time for a number of nucleotides, nucleosides and bases. As can be seen, adenosine had retention time of 12.4 min, while AMP and its other degradation products gave quite different retention times.

The adenosine concentrations were quantitated from peak height measurements and calculated from standard runs. Each day 3 to 5 samples of known amounts of adenosine were analysed prior to measurements of tissue extracts. In each instance, adenosine was dissolved in the extraction blank used for the fluorometric analyses. Fig. 2 shows that there was a linear correlation between adenosine concentration in standard samples and peak height.

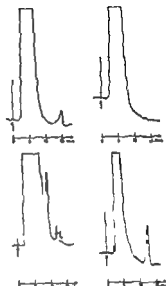
It has been shown by *in vitro* experiments that adenosine is released from cerebra upon electrical stimulation, and following hypoxia or hypoglycemia (Pull and 1972, 1973). Corresponding *in vivo* studies indicate that electrical stimulation or cause increases in tissue adenosine concentration (Rubio *et al* 1975). Adenosine is from AMP in a reaction catalyzed by 5'-nucleotidase (E.C. 3.1.3.5). The rate of this appears to be determined by substrate availability *i.e.* by the AMP concentration *et al* 1972, see also Pull and McIlwain 1973). This raises the question whether an increased tissue concentration of adenosine can be expected always to be present in it accompanied by pronounced vasodilation, *e.g.* hypoxia and epileptic seizures. Thus, data demonstrate that even relatively marked hypoxia is unaccompanied by a decrease in AMP concentration (Bachelard *et al* 1974, Norberg and Siesjö 1975), and epileptic seizures in paralyzed and well oxygenated animals lead to a very small amount of AMP if any (Chapman *et al* 1977). There are two possible explanations for the results: (1) previous *in vivo* data on adenosine concentration are influenced by changes due to inadequate tissue fixation, or (2) adenosine is a more sensitive indicator change in phosphorylation state than AMP.

The present study was undertaken to estimate cerebral cortical adenosine concentration using *in situ* freezing techniques that cause minimal autolytic changes, and to study changes in adenosine, AMP and cyclic AMP in ischemia of maximally 60 s duration. It will be shown that normal adenosine concentrations are considerably lower than previously reported and that there is a strict correlation between changes in adenosine and shortlasting ischemia.

Experimental procedures

Preparation of tissue. Male Wistar rats (275–350 g), with free access to water and rat pellets *ad libitum* were used in the experiments. Anaesthesia was induced with 2% halothane to allow tracheostomy. Animals were then immobilized with tubocurarine chloride and ventilated with 70% N₂O and to keep arterial P_{O₂} above 100 mmHg and arterial P_{CO₂} between 35 and 40 mmHg. One femoral artery was cannulated for continuous recording of the blood pressure and anaerobic sampling of arterial blood analyses. The body temperature was kept close to 37°C. In some of the animals, a parietal craniotomy was performed (see below). Following operation, all animals were allowed a steady state period of at least 15 min.

Control levels of metabolites were obtained from three groups of animals. In group A a skin incision was made and the skull bone exposed to accommodate a plastic funnel for *in situ* freezing of the brain in liquid nitrogen (Pontén *et al* 1973). It has been shown that, with this freezing technique, the cerebral tissue underlying the exposed skull bone freezes in 10–20 s, however, since unfrozen parts of the tissue are continuously supplied with well oxygenated blood, autolytic changes seem to be avoided (Pontén *et al* 1973). In group B a parietal craniotomy (8 × 8 mm) was performed on the left side, exposing the dura. During the steady state period (see above) the dura was kept moist with prewarmed, isotonic NaCl-solution. Brains from these animals were frozen by supercooling the dura with isopentane® precooled to its point of solidification (–156°C), allowing freezing of the superficial 1 mm of the cerebral cortex in about 1 s (Nilsen 1975). On analysis, it was found that there was a pronounced variability in adenosine concentration since corresponding variability was observed in other labile metabolites as well (ADP, AMP and cAMP). It was concluded that operative trauma was involved. Therefore, a new series (group C) was conducted by four different operators who took special precautions not to traumatize the tissue. These precautions included the use of a low-speed burr which was applied with intermittent cooling of the bone by micro-irrigation with a NaCl-solution, and the use of an operating microscope for dislocating the bone flap. In group B, separate animals were decapitated, and the tissue was frozen with isopentane after 30, 40, and 60 s of ischemia. Since some of these brains must have had a preischemic increase in ad-



1. Four chromatograms (for conditions see Fig. 1) showing: A. Extraction blank, stored for several hours at room temperature. Disturbing peaks at 13 and 147 mm can be seen. B. Fresh extraction blank. Here the disturbing peaks are almost eliminated. C. Extraction sample from one of the control animals. A small disturbing peak (\uparrow) is visible in relation to adenosine peak (\uparrow). D. Adenosine in a standard solution prepared in the extraction blank.

retard addition technique which should reduce the error to about +2%. It should be noted that the errors calculated above represent upper estimates since they are based on *total area quantitation*. The errors should be smaller when *band peak height quantitation* is used.

Adenosine concentration in control brains

Table I gives the values for mean arterial blood pressure and body temperature, as well as arterial P_{CO_2} , P_{O_2} and pH, in the three groups studied (see Methods). These physiological variables were very similar between the groups, excluding the possibility of an effect on cerebral metabolism.

Cerebral metabolites are given in Table II. In group A, i.e. in animals whose brains were frozen through the intact skull bone, concentrations of PCr, ATP, ADP and AMP were similar to those previously reported from the laboratory (e.g. Norberg and Siesjö 1975). Cyclic AMP concentrations are similar to those given in a recent report (Fol-

Table I. Physiological parameters in the three control groups (group A: brain tissue frozen through intact skull bone, groups B and C: through exposed dura). Values are given as mean \pm S.E.M.

	Group A (n = 10)	Group B (n = 6)	Group C (n = 18)
Mean arterial blood pressure, mmHg	160 \pm 4	140 \pm 4	130 \pm 3
Temp. $^{\circ}$ C	37.1 \pm 0.1	37.3 \pm 0.3	37.4 \pm 0.1
P_{aCO_2} , mmHg	40.0 \pm 1.0	37.6 \pm 0.7	38.9 \pm 0.7
P_{aO_2} , mmHg	124 \pm 5	133 \pm 5	123 \pm 3
pH	7.393 \pm 0.012	7.370 \pm 0.014	7.388 \pm 0.006

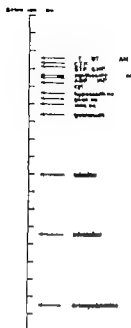


Fig. 1 Retention data for a number of nucleotides, nucleosides and bases (Adenosine at 12.4 min.)

Conditions:

Column 1/4 1 μ Bondpak C18
 Solvent: 90% $(\text{NH}_4)_2\text{HPO}_4$ } pH 7.1
 10% MeOH
 Sample 100 μ l
 Flow rate 1.0 ml/min
 Detector UV 254 nm, 0.0025 AUFS.

Two problems were encountered in the analysis of adenosine. First, if the extract solution was stored at temperatures above -20°C , two peaks (Fig. 3 A) with retention times very close to that of adenosine developed (at 13 and 14.7 min). Tests with exclusion of EDTA or imidazole from the solution indicated that the peaks were due to oxidation products of these substances. In fresh extraction solutions, these peaks were almost undetectable (Fig. 3 B), the peak at 14.7 min being about one third of that due to adenosine in extracts from control brains (Fig. 3 C). The error due to the latter peak can be estimated to about +3% (Snyder and Kirkland 1974). The peak at 13 min, which was indistinguishable from that due to adenosine, explains why the line of Fig. 2 did not pass through the origin. To avoid interference by this peak, the standard solutions were prepared in extraction buffer of equal age as the brain sample extracts (see Fig. 3 D). Second, some of the brain samples were analysed for other metabolites before adenosine was measured. In these samples, a disturbing peak at 14.7 min had reached a height about twice that due to adenosine, giving an estimated error of about -10%. To avoid this error, these samples were analysed with

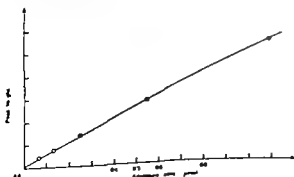


Fig. 2. Correlation between peak height and adenosine concentration in 5 standard solutions. (For conditions see Fig. 1)

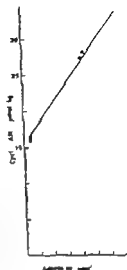


Fig. 5. Relation between tissue concentrations of adenosine ($\mu\text{mol kg}^{-1}$) and cyclic AMP ($\mu\text{mol kg}^{-1}$) in the ischemic animals (16) and the pooled control values (squares represent mean \pm S.E.M. from 28 control animals). Equation of the regression line: Cyclic AMP ($\mu\text{mol kg}^{-1}$) = $0.15 \text{ adenosine} (\mu\text{mol kg}^{-1}) + 1.32$ ($r = 0.94$, $p < 0.001$).

The data show a linear relationship between AMP and adenosine concentrations with a high degree of correlation ($r = 0.99$, $p < 0.001$). The correlation shows that an increase in AMP concentration of $0.017 \mu\text{mol g}^{-1}$ was associated with a doubling of adenosine concentration from 1 to $2 \mu\text{mol kg}^{-1}$.

Fig. 5 gives the relationship between these concentrations of adenosine and cyclic AMP in the ischemic animals (16 animals) and the pooled control values (28 animals). There was a linear relationship between adenosine and cyclic AMP concentrations up to adenosine concentrations of about $10\text{--}15 \mu\text{mol kg}^{-1}$. Experiments with longer periods of ischemia (not shown) indicated that although there was a marked further increase in adenosine concentration the cyclic AMP concentration did not increase above $3.5 \mu\text{mol kg}^{-1}$.

Discussion

The present results are pertinent to questions regarding the normal tissue concentration of adenosine, and relationship between adenosine, AMP and cyclic AMP. These questions will be discussed in turn.

Tissue adenosine concentrations in control situations

It is well known that, in brain tissue, degradation of AMP occurs by deamination to IMP and phosphohydrolysis to adenosine, catalyzed by AMP-deaminase (E.C. 3.5.4.6) and 5-nucleotidase (E.C. 3.1.3.5), respectively (cf. Deuticke *et al.* 1966, McIlwain and Bachelard 1971). The further metabolism of IMP and adenosine leads to formation of inosine in reactions catalyzed by 5-nucleotidase and adenosine deaminase (E.C. 3.5.4.4), respectively. In brain tissues, a perturbation of the phosphorylation state with an increase in AMP concentration leads to increases in adenosine, IMP and inosine; however results obtained on

TABLE II Cerebral cortex concentrations of labile metabolites in animals whose brains were frozen through the intact skull bone (group A) or through the exposed dura (groups B and C). In group C special precautions were taken to avoid trauma to cerebral cortex. Values are means \pm S.E.M. in $\mu\text{mol g}^{-1}$ except for adenosine and cyclic AMP that are given in $\mu\text{mol kg}^{-1}$. In group A and C lactate and pyruvate concentrations were measured in only 4 and 12 animals, respectively

	Group A (n=10)	Group B (n=6)	Group C (n=18)
PCr	4.56 ± 0.06	3.75 ± 0.11	4.12 ± 0.07
ATP	2.96 ± 0.02	2.82 ± 0.04	2.89 ± 0.03
ADP	0.284 ± 0.003	0.333 ± 0.014	0.277 ± 0.005
AMP	0.043 ± 0.004	0.056 ± 0.009	0.044 ± 0.001
E.C.	0.944 ± 0.001	0.931 ± 0.004	0.943 ± 0.001
Lactate	1.58 ± 0.06	2.51 ± 0.42	1.73 ± 0.09
Pyruvate	0.116 ± 0.008	0.123 ± 0.013	0.123 ± 0.006
Lac/Pyruv	13.7 ± 0.7	20.8 ± 4.8	14.1 ± 0.5
Adenosine	0.9 ± 0.1	2.9 ± 1.0	1.1 ± 0.1
Cyclic AMP	1.58 ± 0.09	1.73 ± 0.08	1.62 ± 0.05

$-p < 0.05$ $-p < 0.01$ and $-p < 0.001$ E.C. = adenylate energy charge.

bergrová *et al* 1977) The adenosine concentrations were one third to one fifth of those reported by Rubio *et al* (1975) in the same species.

In group B comprising brains frozen through the exposed dura, the mean adenosine concentration was almost three times as high, with a large scatter ($0.6\text{--}6.7 \mu\text{mol kg}^{-1}$). A corresponding variability was observed for ADP ($0.284\text{--}0.382 \mu\text{mol g}^{-1}$), AMP ($0.039\text{--}0.097 \mu\text{mol g}^{-1}$), lactate ($1.6\text{--}4.2 \mu\text{mol g}^{-1}$) lactate/pyruvate ratio ($11.0\text{--}31.5$), and adenylate energy charge ($0.917\text{--}0.941$). Since it was suspected that this variability was caused by tissue trauma in part of the material, the experiments were repeated with special precautions to avoid trauma (see Methods). The results of this series (group C) corroborated the suspicion since values for labile phosphates were now similar to those obtained in group A. Most importantly the adenosine concentration was close to $1 \mu\text{mol kg}^{-1}$.

Correlation between adenosine, AMP and cyclic AMP in short-lasting ischemia

The relationship between AMP and adenosine concentrations, as obtained in 17 animals subjected to complete cerebral ischemia from 10 to 60 s, is given in Fig. 4. The control values (28 animals) from groups A and C were pooled and are given as a single point (open circle).

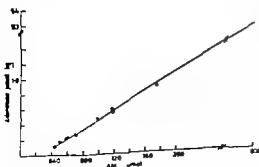


Fig. 4 Relation between individual values of adenosine ($\mu\text{mol kg}^{-1}$) and AMP ($\mu\text{mol g}^{-1}$) in 17 animals subjected to complete cerebral ischemia from 10 to 60 sec. The control values are given as a single point (open circle) representing mean from 28 animals (groups A and C). The regression line, thus representing individual data from 45 animals, is given by the equation: adenosine ($\mu\text{mol kg}^{-1}$) = $0.06 \text{ AMP } (\mu\text{mol kg}^{-1}) + 1.7$ ($r = 0.99$, $p < 0.001$).



Fig. 5 Relation between tissue concentrations of adenosine ($\mu\text{mol kg}^{-1}$) and cyclic AMP ($\mu\text{mol kg}^{-1}$) in the ischemic animals (16) and the pooled control values (square represents mean \pm S.E.M. from 28 control animals). The equation of the regression line: Cyclic AMP ($\mu\text{mol kg}^{-1}$) = 0.15 adenosine ($\mu\text{mol kg}^{-1}$) + 1.52 ($r=0.84$, $p<0.001$).

The data show a linear relationship between AMP and adenosine concentrations with a high degree of correlation ($r=0.99$, $p<0.001$). The correlation shows that an increase in AMP concentration of $0.017 \mu\text{mol g}^{-1}$ was associated with a doubling of adenosine concentration (from 1 to $2 \mu\text{mol kg}^{-1}$).

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rat, rabbit and cat brains indicate that the pathway via adenosine is the most active (Deuticke *et al* 1966, Matthias and Busch 1969, Kleihues *et al* 1974). Recent results suggest that the rate of production of adenosine in the 5'-nucleotidase reaction is mainly determined by substrate supply (Kluge *et al* 1972). Thus it can be expected that whenever there is an increase in AMP concentration the adenosine concentration will rise and, since the concentration of adenosine is less than 5% of that of AMP, it may be a sensitive indicator of change of phosphorylation state.

In view of what has been discussed above it seems clear that true *in vivo* concentrations of adenosine can only be obtained if the fixation technique gives minimal autolytic artefacts. Freezing through the intact skull bone has been shown to give optimal concentrations of phosphocreatine and adenine nucleotides (Pontén *et al* 1973). However, since freezing of the tissue is slow there is the possibility of changes in labile metabolites due to different effects of hypothermia on reactions leading to production and further metabolism of individual metabolites (see results reported on phosphorylase by Folbergrova *et al* 1977).

Freezing through the exposed dura avoids a prolonged period of hypothermia (Nilsen *et al* 1975). However, as the present results show, craniotomy easily leads to minor tissue damage, and to changes in the concentrations of some labile metabolites. Only if special precautions are taken to avoid tissue damage is it possible to obtain values comparable to those measured after freezing through the intact skull bone. The results then obtained demonstrate that the *in vivo* concentration of adenosine is close to $1 \mu\text{mol kg}^{-1}$.

There are few previous measurements of tissue adenosine concentrations, and in such studies the methods have not been well suited to measure concentrations of the order $1 \mu\text{mol kg}^{-1}$ (Deuticke *et al* 1966, Matthias and Busch 1969, Kleihues *et al* 1974). In the experiments of Rubio *et al* (1975), a craniotomy was performed and the brain was frozen using small bone rongeurs precooled in liquid nitrogen. This probably explains why our values were in the range 3 to $5 \mu\text{mol kg}^{-1}$ (cf. results on group B, Table II). This conclusion is corroborated by the high values for lactate concentration and lactate/pyruvate ratio reported by these authors (see also values for AMP in Fig. 6 in Rubio *et al* 1975).

Relationship between adenosine, AMP and cyclic AMP concentrations

The present results corroborate the results of Rubio *et al* (1975) in showing a linear correlation between increases in AMP and adenosine concentrations. The combined results are in line with the conclusion of Kluge *et al* (1972) that the rate of adenosine production in the 5'-nucleotidase reaction is determined by the concentration of AMP. In other words, there are no reasons to assume that adenosine should accumulate in the absence of an increase in AMP concentration. However, since a 50% increase in AMP is associated with a doubling of adenosine concentration, it is possible that increases in extracellular adenosine concentrations of physiological importance can occur in situations, e.g. hypoxia, in which changes in AMP concentrations escape detection (see Introduction). This problem can now be studied by means of analyses of tissue concentrations of adenosine, using adequate *in vivo* freezing techniques. Comparable problems arise in interpreting the correlation between increases in adenosine and cyclic AMP concentrations.

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Capillary Permeability to Albumin in Normotensive and Spontaneously Hypertensive Rats

By

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Abstract

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Transcapillary passage of plasma proteins is enhanced in man in primary hypertension and it is debated whether this reflects increased permeability or merely a raised capillary pressure. To elucidate this problem maximally vasodilated hindquarters of spontaneously hypertensive rats (SHR) and normotensive control (NCR) were perfused in parallel at constant flow with dextran, horse serum or mixtures of the two, with labelled albumin as indicator of capillary permeability to macromolecules. By equal increases of capillary pressure modest filtration was maintained during one hour after which the edema and its albumin content were determined. — There was less edema in SHR reflecting a slightly lower postcapillary resistance and a much higher precapillary resistance compared with NCR, which here resulted in a lower capillary pressure. In SHR, in both SHR and NCR the presence of dextran slightly enhanced the capillary filtration coefficient but increased albumin permeability up to tenfold, also after antihistamine drugs. However for each perfusate the SHR capillaries were, if anything, slightly less permeable to albumin than the NCR ones. — The results suggest that the enhanced transcapillary passage of plasma proteins in primary hypertension reflects an increased capillary pressure in some circuit(s), probably mainly skeletal muscle, resulting from the functional balance in / between the pre- and postcapillary resistances.

Quantitative studies concerning design and principal hemodynamic characteristics of the different series-coupled vascular sections have been sparse in primary hypertension, particularly when compared to the situation in normotension. However such a study was recently performed by Folkow *et al* (1974) in spontaneously hypertensive rats (SHR Okamoto 1969), in comparison to normotensive control rats (NCR). The results showed that the structural increase in wall/lumen ratio generally characterizing large arteries and resistance vessels in primary as well as secondary hypertension (*cf* Folkow *et al* 1973), is largely confined to the precapillary vessels, at least in the hindquarter section of the systemic circulation. Thus, the capillary section in the preparation used seemed to be essentially unchanged in SHR, insofar as the "isogravimetric" capillary pressure and also the capillary filtration coefficient (CFC) values were the same as in NCR at maximal dilatation. The postcapillary resistance vessels appeared to be, if anything, structurally slightly wider in the SHR than in the NCR hindquarter vascular bed and they exhibited no sign of any "hyperreactivity".

constrictor or dilator agents, a response which characterizes the precapillary vessel outflow (Hill *et al.* 1974). Such observations indicate that the increased precapillary resistance in hypertension "protects" the exchange and postcapillary venous vessels from the impact of the increased arterial pressure, though the situation may later change if *e.g.* a failing heart leads to an increased central venous pressure which is transmitted in the retrograde direction. It was, however, recently discussed by Parving (1975) whether capillary permeability might be increased already in early phases of essential hypertension in man, in respects that are not necessarily reflected in CFC measurements. This proposal was mainly based on the fact that already in early essential hypertension there occurs an increased transcapillary escape rate (TER) of intravenously injected labelled albumin (*cf.* Parving and Gyöteberg 1973, Urych 1973). Capillary permeability is, however, only one of the factors determining the rate of transcapillary exchange of plasma proteins, another important factor being the level of capillary pressure. Thus, there is good evidence that elevations of capillary hydrostatic pressure increase the net protein escape across the exchange vessels (*e.g.* Landis *et al.* 1932, Szabo, Magyar and Papp 1963, Haddy, Scott and Greig 1972, Lassen, Parving and Roming 1974).

For such reasons the increase of TER, observed in subjects with essential hypertension, may simply be due to a raised capillary pressure. Urych (1973) has, for example, discussed whether an increased TER might ensue as a result of vasoconstriction particularly involving the venous side. However, it is in this connection of considerable relevance that muscle blood flow is commonly enhanced in essential hypertension (*cf.* Pickering 1968), particularly in its early "hyperkinetic" phases (*cf.* Julius and Shorr 1971) simulating the hemodynamics of very light exercise or perhaps better in a mild defence reaction (*cf.* Folkow and Neil 1971, Julius and Esler 1975). In such situations the precapillary resistance of the muscle circuit is lowered compared with most other circuits, while venous capacitance tone is, if anything, increased. Particularly when associated with an increased arterial pressure, this most likely implies a substantial enhancement of capillary pressure in skeletal muscle tissue. Obviously the Starling equilibrium in this large tissue mass is then shifted towards net filtration and with this follows an increased capillary transfer of plasma proteins, as during normal exercise. A quite recent study (Rippe, Kamya and Folkow 1977) provides strong evidence against the often proposed view that macromolecules should mainly pass capillary walls by way of micropinocytosis in such situations.

In experiments on man it is very difficult to distinguish clearly between *e.g.* a slight deviation in the capillary permeability-surface characteristics (PS product) and one in mean capillary pressure, particularly when comparing mild, uncomplicated cases of essential hypertension with normotensive controls. Therefore, if primary hypertension really involves a deviation in capillary PS characteristics, the chances of revealing such a change would be better if animal models of primary hypertension are utilized instead. Since this problem is one of principal interest in the evaluation of hypertension in general, it was considered worthwhile to perform a quantitative comparison between SHR and NCR hindquarters with regard to their capillary permeability to proteins under strictly controlled experimental conditions. This was done, keeping the entire capillary bed open to flow while maintaining other hemodynamic factors as equal and constant as possible during paired SHR-NCR

perfusion with plasma and different plasma substitutes, containing labelled albumin tracer of transcapillary protein transfer

Methods

Technically successful experiments were performed on 79 pairs of Wistar spontaneously hypertensive (SHR; Okamoto 1969) and weight matched Wistar normotensive control rats (NCR). All rats were sex and weighed between 290–410 g. The majority of them were 6–9 months old and hence in an early or middle phase of established hypertension. The isolated hindquarters of one SHR and weight matched NCR were simultaneously perfused at constant rate and at essentially equal flows. Outflow pressures were moderately elevated but to an equal extent in the two animals, to induce a capillary filtration and consequent transfer of plasma proteins.

Preparation. Initially mean arterial blood pressure (P_A) was measured in the awake experimental rat in a polyethylene cannula, inserted into the tail artery during brief ether anaesthesia, utilized for recordings throughout the experiment via Statham P23 AC transducer writing on a Grass Pot. Afterwards the animals were anaesthetized with Nembutal 3 mg/100 g administered into the tail cannula, simultaneously eviscerated and the abdominal aorta and caval vein were freed between the ilio-lumbar vessels. The hindquarters were otherwise completely isolated by standardized cannulae at identical levels just proximal to the ilio-lumbar vessels, and the tail distal to the tail artery cannulae paws were excluded so that the hindquarter preparation mainly consisted of skeletal muscle (cf. *et al.* 1970).

After heparinization the abdominal aortas in both rats were ligated, immediately cannulated in the direction and connected to a common perfusion system which was already running. The two canals were then ligated, immediately cannulated and connected to 30 cm long outflow tubes, after which the animals were killed. The pressure drop along the two venous outflow tubes was equal and about 5 mmHg at a perfusion rate of 10 ml/min. Their outflow tips could be raised or lowered about 25 cm and thus that venous outflow pressures of the two preparations were always identical the tips were linked together.

To induce moderate filtration, venous outflow pressures (P_V) were elevated to an identical extent in two preparations, usually from 6 up to 18 mmHg. In some of the paired experiments venous pressure was just proximal to the outflow tubes were measured directly in the cannulae in venous side branches and found to be identical at given levels of outflow pressure. The artificial perfusion system utilized in both animals, as described in detail earlier (cf. Folkow *et al.* 1970).

Perfusates. It is known since long that perfusion of vascular beds with dextran affects capillary barrier characteristics, considered to be mainly due to the absence of native proteins (cf. Lands and Fjelmer 1963; Areekul 1969). To investigate the problem further in these comparisons of normotensive and hypertensive vascular beds, 4 different perfusates were employed, i.e. horse serum (Normal SBL, Sweden), dextran (Macrodex®-70; MW about 70,000, generously supplied by AB Pharmacia, S-413 45 Mölndal, Sweden) and two different mixtures of dextran and horse serum. All colloids were mixed with Tyrode solution the perfusates were kept at 38°C and oxygenated. Because of the differences in perfusate the animals were divided into 4 groups.

In **group I** all matched pairs of NCR/SHR were perfused with horse serum (containing 65 g of protein/liter), diluted with Tyrode to 60 per cent of its initial concentration, i.e. a 39 per cent content of protein.

In **group II** eight matched pairs of NCR/SHR were perfused with 4% dextran in Tyrode.

In **group III** eight matched pairs of NCR/SHR were perfused with dextran in Tyrode of the same concentration as in group II but with addition of 5 g/l of horse serum protein.

In **group IV** seven matched pairs of NCR/SHR were perfused with a mixture of dextran-Tyrode (g/l) and native horse serum, in the proportions of 2:1. The final dextran concentration was then 2% and the protein concentration about 2%.

Control stage of perfusion. The vascular beds were initially "flushed" for 2–3 min by adjusting pump to deliver 15–20 ml/min 100 g tissue in order to wash out all traces of blood. To induce vasodilatation, repeated doses of papaverine, 1–2 mg (a time), were given to both hindquarters in a common mixing chamber in the perfusion system until no further pressure drop occurred. This state of vasodilatation was kept stable throughout by repeated papaverine injections or papaverine infusion.

During the subsequent 10–15 min flow was kept low at 3–5 ml/min 100 g tissue, while venous pressure was near zero, as indicated by the readily visible collapse of both venous trees. The hindquarter capillary bed is then slightly absorbing, as observed earlier (Folkow *et al.* 1974) and confirmed in a

here the handquarterers were continuously weighed. This was done to avoid initial edema formation that the preparations would preferably have slightly reduced interstitial fluid space. At the end of this run, flow are increased identically and varied rapidly during few minutes in order to obtain data constructing flow-pressure curve for each preparation, which better characterizes the dimensions their resistance vessels. Some filtration was thereby induced, more or less reversing the initial fluid loss, as shown from experiments here the handquarterers are continuously weighed.

Perfusion at arteriole After these initial stages of perfusion, tracer quantities ($1 \cdot 10^{-4}$ g/l) of 125 I-Human serum Albumin (Radiochemical Center Amersham England; γ -radiation energy 0.035 M V) are added to the perfusate. In the two venous outflow cannulae were raised identically to induce filtration. Flow as then kept around 9 ml/min. 100 tissue and equal in the two preparations. This period of perfusion its addition of labelled protein to the perfusate was continued for 60 min, the venous effluents being collected and, after draining and reoxygenation, recirculated once or twice. Arterial pressure was continuously checked and it was ensured that maximal vasodilatation was throughout maintained in both preparations. Thus, the entire capillary surface area was exposed to flow throughout and at moderately low pressure levels because of the increased venous outflow pressure.

The specific activity of the perfusate was determined repeatedly (see below) and its radioactivity fell about 2-3% in 1 h because of the recirculations. Non-protein bound radioactivity of the perfusate, as determined before and after the perfusion period as specific activity of the supernatant, following precipitation of the perfusate proteins with 10% trichloroacetic acid. It varied from 1.0-1.8 per cent of total perfusate radioactivity but did not change in the course of given experiment.

After about 60 min of perfusion with the tracer-containing perfusate, rapid shift to perfusion with tracer-free, but in other respects identical perfusate was performed. This tracer-free perfusion was stopped when the specific activity of the venous effluent is still below 3 per cent of the arterial activity which usually occurred after 7-8 min. During this final tracer-free perfusion period, the venous outflow cannulae were lowered to the level of the aorta in order to minimize further filtration.

Determination of tracer transfer After stopping the tracer-free perfusion, three separate muscle groups from each of the two handquarterers were dissected out bilaterally namely 1) the anterior crural group as tibialis ant. m. extensor dig. longus and m. extensor hallucis proprius; 2) m. gastrocnemius and soleus, 3) the larger part of m. quadriceps. Immediately after dissection each muscle preparation was put into ice water. After scaling and weighing, the tube was put into well covered, connected to Packard Autogamma Spectrometer (Model 410 A), for determination of radioactivity by counting minimum of 10 000 c.p.m. Each muscle was then completely dried at 90-100°C for 7 h and re-weighed.

Wet weights (W_0) and dry weights (D) of corresponding muscle groups from 6 MCR and 6 SHR, not used for experiments were determined separately as controls. The SHR and MCR muscles did not differ markedly concerning the relationship of dry weight/wet weight. The following normal values for the muscle muscle groups, expressed as per cent of their wet weights ($D/W_0 \cdot 100$), were determined and used as controls: 1) the anterior crural muscle group 4.7 per cent, 2) m. gastrocnemius-soleus 24.2 per cent, 3) m. quadriceps 23.6 per cent. Knowing these percentages ($D/W_0 \cdot 100$) for muscles from non-perfused control animals, together with the dry weights and wet weights after perfusion (W) from the muscles of the experimental groups, the edema content ($W_0 - W$) of the "experimental" muscles, expressed in g per 100 g of tissue, could be determined. Muscle radioactivity corrected for background activity was then divided by the edema content ($W_0 - W$) and by the specific activity of the perfusion medium. Correction for free iodine activity was not deemed necessary because the free iodine is supposed to be distributed extracellularly and should thus easily be washed out during the 5 min of final tracer-free perfusion.

In this way the ratio between the albumin concentration of the newly formed edema fluid (C_p) and that of the perfusate ("plasma") (C_p) is obtained for every muscle group and animal. All the experiments, especially those of group 1, were performed under conditions of moderately enhanced capillary filtration. The purpose was to create a situation in which the transcapillary albumin flux was largely filtration dependent, while absorption and net diffusion could be neglected (concerning macrophagocytosis see Ruyss, Karmy and Fallow 1977). Furthermore, escape by means of lymphatic flow could also be ignored since essentially all lymphatic vessels were blocked by ligation during preparation. The newly formed edema must then be largely equal to the filtrate formed during the experimental period.

Under these conditions the filtrate/plasma concentration ratio of albumin, C_p/C_p , will approach A_p/A_w . Let the ratio of "effective" pore area available to albumin to that of water. The same deduction can be made from the relation developed by Pappenheimer (1953) $C_p/C_p = (PS \cdot F \cdot A_p/A_w) / (PS + F)$, where C_p/C_p is the filtrate/plasma concentration ratio and PS the diffusion capacity for the macro-molecule, while F is the filtration rate. At high filtration rates C_p/C_p will be good approximation of A_p/A_w , and thus fairly

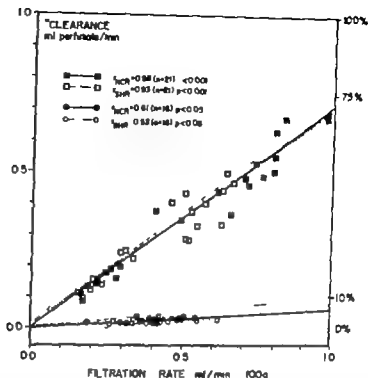


Fig. 1 Relationship between perfusate clearance of albumin and filtration rate for NCR and SHR groups I (circles: 60% horse serum and 40% Tyrode) and II (squares: 4% dextran in Tyrode). As seen dextran perfusion implies a tenfold increase in albumin clearance but without any appreciable difference between NCR and SHR. Further for each of the perfusates shown in the Fig. C_p/C_p remains essentially constant independent of the filtration rates used, which was also the case for the other two perfusates (groups I and IV). — Each point represents one out of three duplicate determinations per animal, performed on the different muscle groups which varied somewhat concerning extent of edema formation.

constant. In the present experiments C_p/C_p could thus be used as a measure of capillary permeability to albumin. At the prevailing filtration rates there was namely a good correlation between the "plasma" clearance of albumin and filtration rate in all groups, i.e. almost constant C_p/C_p ($\approx A_p/A_p$) was obtained.

Statistical evaluation was performed by the paired design *t* test: mean values of the six determinations of C_p/C_p in every SHR being compared with the corresponding NCR values. To obtain comparisons between the differently perfused groups, the groups comparison *t*-test was utilized. At *p* values below 0.05 the differences were considered significant.

Results

A Filtrate to "plasma" albumin concentration ratios (C_p/C_p)

1 C_p/C_p at different filtration rates and different "plasma" compositions. A good correlation was found between the rate of edema formation and net transcapillary outflux of albumin in all animal groups. Independent of the filtration rate (which varied between 0.1–1.0 ml/min 100 g depending on the venous pressure level as related to the respective levels of isogravimetric capillary pressure for the different perfusates) the concentration of protein in the filtrate remained almost constant for a given perfusate. This was evident by the fact that the concentration of tracer albumin in the filtrate was that in the perfusate did not change appreciably with increasing filtration and this was so for both NCR and SHR (Fig.

CAPILLARY PERMEABILITY TO ALBUMIN IN NCR AND SHR

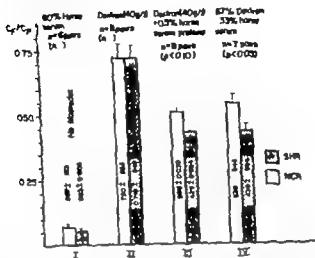


Fig. 2. Ratios of perfusate concentration ratios of labeled albumin (C_p/C_p) in NCR and SHR during perfusion with horse serum (group I) or dextran-containing perfusates (groups II-IV see text). Each observation on a given animal represents mean value from three duplicate determinations, for every new group of experimental animals means \pm S.E. are given in the figure. — Note that C_p/C_p increases markedly when the perfusate contains dextran but that the SHR capillaries are, if anything, less permeable to protein than the NCR ones for each perfusate used.

using perfusion with horse serum-Tyrode (group I) the percentage of tracer protein filtrate versus "plasma" was as low as 3-7% in both NCR and SHR (see also Fig. 2). On 40 g/l of dextran (Macroderm) in Tyrode was employed as perfusate (group II), C_p/C_p increased tenfold, being 0.70-0.75 in both NCR and SHR, but if small amounts of horse serum proteins (5 g/l) were added to this dextran perfusate (group III), C_p/C_p decreased to 3-0.5. However, as long as dextran was present no further reduction of C_p/C_p was seen as the protein concentration was increased up to 20 g/l (group IV).

C_p/C_p in SHR compared with NCR. Mean values of C_p/C_p in the six SHR hindquarters perfused with horse serum (group I) was 0.053 ± 0.008 compared with 0.067 ± 0.013 in the matched NCR (Fig. 2), values which closely agree with findings in blood perfused cat aorta (Appelgren, Jacobsson and Kjellmer 1966). Although NCR here showed a somewhat higher C_p/C_p than SHR in 5 expts. out of 6, the difference was not statistically significant. Anyway it seems safe to conclude that SHR does not show any higher albumin permeability than NCR. The results are in agreement with earlier findings concerning identical capillary filtration coefficients in SHR and NCR hindquarters at maximal dilatation (Folkow *et al.* 1974), confirmed in other more recent measurements (unpublished).

During perfusion with dextran alone (group II) C_p/C_p increased, as mentioned, about tenfold in both NCR and SHR, and this was so also in preparations given large amounts of an antihistaminic agent. However, under these conditions of a very marked increase in capillary membrane permeability to albumin, C_p/C_p was identical in SHR and NCR being 0.70 ± 0.05 (mean \pm S.E.) in both. After addition of proteins to the dextran perfusate, first 5 g/l (group III) and then 20 g/l (group IV) mean C_p/C_p values were reduced to 0.51 ± 0.04 in NCR and to 0.43 ± 0.03 in SHR (15 pairs) with no significant differences between groups.

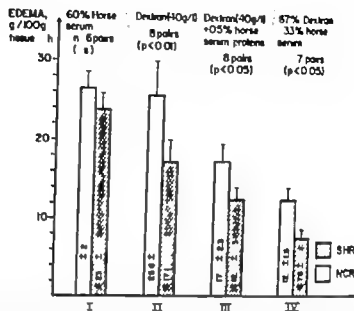


Fig. 3 Amount of edema (mean \pm S.E.) formed during one hour of perfusion with the four perfusates. Although capillary pressure was kept largely equal in all groups, 17–18 mmHg in NCR, effective filtration pressure varied between the four groups due to differences in colloid osmotic pressure; further, CFC 6 differed modestly with the perfusate (see text). In NCR effective filtration pressure, as estimated to 14 mmHg in group I and some 9–7 and 5 mmHg, respectively, in groups II, III, IV, the corresponding π being 1–2 mm lower in SHR.

III and IV. However, here the slightly lower values in SHR differ significantly from those in NCR (Fig. 2).

B. Edema formation

1. *Differences in edema formation rate in the various groups.* The edema formation during 1 h of tracer perfusion varied significantly between the 4 groups (Fig. 3). The explanation is partly that effective filtration pressure, i.e. the difference between the prevailing net hydrostatic pressure in the capillaries (P_c) and the isogravimetric one (P_{cl}), differed between the groups depending on the perfusates used.

In NCR, mean P_c was estimated to 17.7 ± 0.4 mmHg during the period of raised P_v . In the majority of experiments, as calculated from the prevailing P_A and P_V values and from the pre- to postcapillary resistance ratio (which at these distending pressures is about 3:1 in NCR according to Fig. 1 in the paper by Folkow *et al.* 1974). This P_c value during the filtration period did not differ significantly in the various groups of NCR as tested with variance analysis. The same calculations for SHR in the various groups (where the P_c increases were always identical to the paired NCR and where the prevailing pre- to postcapillary resistance ratio is rather about 5:1 according to Folkow *et al.* 1974) resulted in a mean value for P_c of 16.6 ± 0.4 mmHg at the prevailing P_A and P_V levels. This P_c level in SHR was significantly lower than that in NCR ($p < 0.001$), and the results by Folkow *et al.* 1974 as well as unpublished results with the isogravimetric technique are, in agreement with a true difference in P_c between NCR and SHR when P_V and flow are equal.

From these P_c values the effective filtration pressures can be approximately estimated for the four groups of NCR and SHR. To start with group II (4% dextran) the P_{cl} value

round 9 mmHg for both NCR and SHR, as judged from unpublished "isogravimetric" estimations using the dextran perfusate. This gives an effective filtration pressure of about mmHg in NCR with the SHR value being 1 or 2 mmHg lower which at least in part explains the different edema amounts (Fig. 3).

In group III (4% dextran + 0.5% horse serum proteins) the edema formation was some 10-15% lower than in group II. This is likely to reflect the moderately higher colloid osmotic pressure of this perfusate, in association with some reduction in capillary permeability as caused by the protein addition, and independent CFC measurements show this. However the CFC reduction is only of the order of 20-25%, where the raised colloid osmotic pressure is likely to account for the rest of the difference between groups II and III. A proper fit with the present data is obtained if the 0.5% protein addition raised P_{col} from 9 to about 11 mmHg. If so the effective filtration pressures in group III would amount to about 7 mmHg in NCR and 6 mmHg in SHR (Fig. 3).

In group IV (2.7% dextran + 2% horse serum proteins) the edema formation again decreased significantly presumably due to a further increase in colloid osmotic pressure of the perfusate. 4% dextran exerts colloid osmotic pressure in the skeletal muscle vascular bed only some 16 mmHg (Eliasson *et al.* 1974), and 2.7 per cent dextran mixed with 2% serum proteins, which per unit weight exerts about the same colloid osmotic pressure as dextran, must imply a colloid osmotic pressure that is perhaps 2.5-3 mmHg higher than for 4% dextran. Consequently the effective filtration pressures would here be in the order of 4-5 mmHg in NCR and 2-3 mmHg in SHR.

Finally in group I (3.9% horse serum proteins) the colloid osmotic pressure is bound to be a few mmHg below that in group II, being about 14-15 mmHg and resulting in a P_{col} around 8 mmHg for both NCR and SHR. The effective filtration pressure in NCR would then be 10-11 mmHg and 1-2 mmHg lower in SHR, *i.e.* somewhat higher than for group II, however CFC is lower (20-25%) for horse serum than for dextran, as mentioned above, so the rate of edema formation would be expected to be fairly equal in groups I and II as also shown in Fig. 3. — These estimations of differences in net filtration pressures with various perfusates are admittedly approximate but are likely to be realistic as to their general order. They are necessary to explain the differences in edema formation between the four groups of experiments and also for the comparison between SHR and NCR.

2. Amount of edema in SHR and NCR. Although the paired SHR-NCR hindquarters were always exposed to identical venous pressures, the SHR hindquarters showed throughout lower amount of edema formation than NCR during the 60 min of perfusion, as shown in Fig. 3. Though this difference was quite small and not significant in group I, it was more substantial in the dextran group I ($p < 0.01$) and in groups III and IV ($p < 0.05$) where dextran and horse serum were mixed. These differences can hardly be ascribed to any differences in CFC, since SHR and NCR here show equal values (Folkow *et al.* 1974, unpublished observations). They rather suggest first a lower mean hydrostatic pressure in SHR than in NCR as result of differences in both pre- and postcapillary resistances (*cf.* Folkow *et al.* 1974). Second, there might be a slightly smaller leakage of macromolecules to the interstitial space in SHR (see Fig. 4) which, if anything, would maintain a somewhat higher effective colloid osmotic pressure in the SHR capillaries. In any case, there are

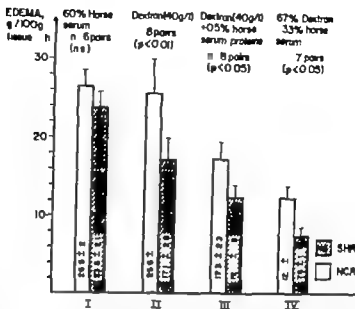


Fig. 3 Amount of edema (mean \pm S.E.) formed during one hour of perfusion with the four perfusates. Although capillary pressure was kept largely equal in all groups, 17–18 mmHg in NCR, effective filtration pressure varied between the four groups due to differences in colloid osmotic pressure. Further CFC differed modestly with the perfusate (see text). In NCR effective filtration pressure was estimated to 10 mmHg in group I, and some 9.7 and 5 mmHg, respectively in groups II, III, IV the corresponding value being 1.2 mm lower in SHR.

III and IV. However, here the slightly lower values in SHR differ significantly from the NCR (Fig. 2).

B Edema formation

1 *Differences in edema formation rate in the various groups.* The edema formation during 1 h of tracer perfusion varied significantly between the 4 groups (Fig. 3). The explanation is partly that effective filtration pressure, i.e. the difference between the prevailing mean hydrostatic pressure in the capillaries (P_c) and the isogravimetric one (P_{ci}), differed between the groups depending on the perfusates used.

In NCR mean P_c was estimated to 17.7 ± 0.4 mmHg during the period of raised P_v in the majority of experiments, as calculated from the prevailing P_A and P_V values and from the pre- to postcapillary resistance ratio (which at these distending pressures is about 3:1 in NCR according to Fig. 1 in the paper by Folkow *et al.* 1974). This P_c value during the filtration period did not differ significantly in the various groups of NCR, as tested with variance analysis. The same calculations for SHR in the various groups (where the P_v increases were always identical to the paired NCR and where the prevailing pre- to postcapillary resistance ratio is rather about 5:1 according to Folkow *et al.* 1974) resulted in a mean value for P_c of 16.6 ± 0.4 mmHg at the prevailing P_A and P_V levels. This P_c level in SHR was significantly lower than that in NCR ($p < 0.001$) and the results by Folkow *et al.* 1974 as well as unpublished results with the isogravimetric technique are, in agreement with a true difference in P_c between NCR and SHR when P_V and flow are equal.

From these P_c values the effective filtration pressures can be approximately estimated for the four groups of NCR and SHR. To start with group II (4% dextran) the P_{ci} value is here

hypertensive crises with serious consequences particularly for the brain (e.g. Johansson 1974). The precapillary autoregulatory response then yields to the overwhelming rise in wall tension, thereby transmitting the greatly increased transmural pressure to the exchange vessels. However the structural precapillary changes typical of hypertension here offer relative protection, since their stronger media does not yield as easily as that of normotensive vessels (cf. Hallböök, Lundgren and Weiss 1974, Johansson 1975).

In any case, the present experiments on an animal model of man's essential hypertension provides no evidence of either any "primary" or "secondary" increases in capillary permeability in SHR, whether in terms of protein permeability or CFC (Folkow *et al.* 1974). It is then likely that the increased TER in essential hypertension (Ulfvich 1973, Purving 1975) effects a hemodynamic situation where instead mean capillary pressure is somewhat raised in at least some of the major systemic circuits. In fact, also young SHR show a tendency of an increased TER in the "resting" awake steady state (Rippe, Lundin and Folkow 1977). As mentioned in Introduction, muscle blood flow is commonly increased in essential hypertension and particularly so in early "hyperkinetic" stages (cf. Julius and Shork 1974), when the hemodynamic pattern simulates that of a mild defence reaction or very light exercise. The muscle vasodilatation in these situations is mainly precapillary in nature, which inevitably implies a raised capillary pressure, and therefore an increased formation of filtrate. With this follows an increased transcapillary albumin escape, apparently mainly by means of increased filtration through large pores (bulk flow), while capillary permeability *per se* in all likelihood remains unchanged (cf. Arturson and Kjellmer 1964). A concomitant presence of a raised central venous pressure due to α venoconstriction will, of course, further tend to enhance the filtration by a retrograde effect on the pressure level in the microvessels.

The present experiments further showed a less pronounced edema formation in SHR compared with NCR (Fig. 3), despite largely equal CFC values and isogravimetric capillary pressures (Folkow *et al.* 1974). Filtrate to plasma concentration ratios of albumin (C_F/C_P) were in most experiments slightly reduced in SHR compared to NCR (about 15%). Therefore capillary permeability to macromolecules is, if anything, lower in SHR than in NCR. As a result the total outflow of labelled albumin into the SHR hindquarter interstitial fluid space was up to some 35% lower in SHR. This indicates that effective filtration pressure must have been throughout lower in SHR than in NCR, in extent varying with the perfusates used as outlined in Fig. 3 and essentially resulting from a different balance between pre- and postcapillary resistances in SHR, since venous outflow pressure and flow were equal. At maximal vasodilatation the SHR hindquarter vascular bed displays a substantially higher precapillary resistance than the NCR one (Folkow *et al.* 1974), but had this been the only difference at equal flows mean capillary pressure would have also been equal. Therefore, both the mentioned study and the present results suggest that postcapillary resistance of the hindquarter vascular bed is slightly lower in SHR than in NCR during complete vascular relaxation. However in the *in vivo* situation, the superimposed neurogenic-myogenic smooth muscle tone may of course, readjust the hemodynamic situation over a wide range when necessary.

The drastic increases in C_F/C_P caused by dextran and only moderately reversed by plasma proteins in the perfusate were in these experiments utilized for comparing the permeability

certainly no signs of any increased capillary permeability in SHR compared with NCR in the present results

Discussion

As outlined in Introduction, studies on man indicate an increased transcapillary exchange rate of albumin (TER) in essential hypertension (Parving and Gynedberg 1973, Ulfberg 1973, Parving 1975). Whether this increased turnover rate is due to an altered capillary permeability to macromolecules in general or simply caused by an increased mean capillary pressure in some major systemic circuits, or to a combination of both, has not been analysed in detail in hypertensive subjects. Parving has discussed the former alternative, while Ulfberg seems to be more inclined towards the latter view.

It is of principal interest both concerning which type of early vascular changes that occur in primary hypertension and for the correct analysis of the hemodynamic situation in this disorder to know whether also the capillary exchange vessels proper exhibit any alterations in permeability. It is, for example, theoretically possible that the genetically linked deviations in cardiovascular design and function might involve a "primary" deviation also in this respect and/or that "secondary" changes may occur early in the development.

Against such a background the present investigation was performed to study selectively albumin permeability and rate of edema formation during induced filtration in the artificially perfused, isolated and maximally dilated hindquarter vascular beds of spontaneously hypertensive and normotensive control rats (SHR, NCR). The essential result was that there are no signs of any increased capillary permeability to albumin in SHR compared with NCR, nor do they differ in capillary filtration coefficient (cf Folkow *et al* 1974). If anything, the SHR hindquarter vascular bed seems to show a slightly lower albumin permeability than that of NCR. Thus, there is no evidence of any "primary" increase of capillary permeability in this type of primary hypertension.

The capillary exchange vessels might as mentioned also display a secondary increase in permeability e.g. if they are in the course of hypertension frequently exposed to large pressure rises. However such a possibility also seems to be excluded by the present findings in SHR, being in the phase of fairly well established hypertension. The main reason is probably the fact that the increased resistance in established hypertension is largely confined to the precapillary vessels (Folkow *et al* 1974), which tends to protect the capillaries from pressure elevations except when the precapillary resistance for functional reasons is relatively reduced. However even when this is the case, as normally occurs in the muscle circuit during exercise or defence reactions, the capillary membranes prove to be quite resistant, at least as long as such pressure rises are within the "physiological" range. Perhaps the most dramatic example is the situation normally facing the lower limb capillaries of man while erect, though precapillary autoregulatory responses, reflex adjustments and the venous "pump" will also here protect the exchange vessels from undue pressure rises.

This by no means denies that acute, marked pressure increases may overcome the protective capillary counterregulatory mechanisms, causing vascular distension, wall edema and at last gross capillary leakage (Giese 1964 a, b), a situation evidently not seldom met with

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properties of the capillaries over a wide range of situations. The nature of these changes in permeability is, however *per se* of interest and worthy of a brief comment already here though the problem will be dealt with in a subsequent study designed to compare simultaneously the filtration and diffusion events (*cf* Rippe and Stage 1976). The decrease in capillary selectivity for macromolecules induced by dextrans has earlier been studied by Areekul in the perfused rabbit ear. At blood perfusion the reflection coefficient for plasma colloids was 0.94 while it was only 0.3 at perfusion with 3% dextran concentrate (mol. w. around 80 000) increasing to around 0.5 after adding 5 g/l of pig plasma protein to the perfusate. These values can be roughly transformed so as to correspond to the present used sieve coefficients (C_p/C_f) by being subtracted from 1 and they are then closely similar to the C_p/C_f values of the present study (*cf* Fig. 2.) Further preliminary results on the isolated rat hindquarter preparation show that CFC increases some 35% by shifting from horse serum perfusion to 4% dextran perfusion *i.e.* from around 0.035 ml/min 100 g (*cf* Renkin and Zaun 1955) to around 0.048 ml/min 100 g at dextran perfusion at complete vascular relaxation. These changes in permeability to pore-bound water filtration are small compared with the nearly tenfold changes in large molecular permeability (0.6 compared with 0.70).

On the basis of CFC determinations it has been claimed that the permeability changes induced by dextran can be almost completely reversed by the addition of proteins to the perfusate (*cf* Landis and Pappenheimer 1963). In the present study the addition of even small amounts of proteins to the dextran perfusate considerably reduced CFC but the albumin concentration in the filtrate remained fairly high even if substantial additions of plasma protein were given. Thus, the presence of dextran molecules seems to more strongly enhance capillary permeability to large molecules than that determining the filtration of water but we will, as mentioned, come back to these problems in another study.

The mechanisms behind these actions of dextran on the capillary membrane are not understood (*cf* Areekul 1969) but the influence on permeability to colloids shows some relationships to that exerted by *e.g.* histamin or bradykinin. Although dextran is known to release histamin from mast cells in rats, infusion of even huge doses of the antihistaminic drug clemastin (Tavegil® Sandoz) in four experiments did not affect the C_p/C_f values significantly. Further, in rabbits histamin is evidently not released in response to dextran, and as the present data closely simulate those found in rabbits (Areekul 1969), implying that histamin is not likely to mediate the mentioned permeability changes induced by dextran. It might represent some type of a direct action of dextran on the pores, since there are reasons to believe that micropinocytosis is not involved in these processes (Rippe, Kamlya and Folkow 1977).

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h cardiac and vascular muscles are dependent, to a great extent, on Ca^{2+} influx from the extracellular environment. In this respect, however, smooth muscle effectors do vary considerably depending on their location along the vascular bed. For example, at Ca_o concentrations as low as 1.0 mM the portal vein is completely unresponsive to noradrenaline (NA), aortic strips retain much of their NA responsiveness. Furthermore, the dependence on Ca_o seems to vary with the type of muscle activation. Thus, upon graded reduction of Ca_o the myogenic activity of the portal vein vanishes well before its responses to NA administration (Folkow *et al.* 1976), suggesting a more efficient mobilization of calcium stores when membrane polarization is induced by the adrenergic transmitter than when it occurs "spontaneously". Recent studies (Folkow *et al.* 1976, Sotter *et al.* 1977) on the responsiveness of the microvascular sections, which are of particular hemodynamic importance because of their control of pre- and postcapillary resistance, capillary flow distribution and of capacitance, indicate an increasing dependence on external calcium the closer one comes to the capillary level. Thus, in this and in several other respects, the smooth muscle of the microvessels is more similar to that of the portal vein than to that of the aorta or pulmonary artery while the more proximal precapillary vessels appear to fall somewhere in between.

The question arises whether chronic hypertension, besides having hemodynamically important effects on precapillary vessels design in terms of increased media thickness in association with a reduced internal radius (*cf.* Folkow *et al.* 1973, 1974), may also involve changes of smooth muscle function in the microvessels in that the process of excitation-contraction coupling is altered, as reflected, *e.g.* in a changed dependence on external calcium. In the past few years this has been a subject of much discussion as to whether a change in this coupling might be decisive for the altered reactivity of the resistance vessels (*e.g.* Bach and Haeusler 1974).

For such reasons, an experimental analysis was performed of the extent to which the resistance responses are affected by Ca_o^{2+} changes in normotensive Wistar rats (NCR) as compared with young and adult spontaneously hypertensive rats (SHR, Okamoto strain) and renal hypertensive rats (RHR, induced by unilateral renal clipping in NCR). Part of the results has been briefly reported in an earlier paper (Folkow *et al.* 1976).

Methods

1. Material

The present study is based on hemodynamic analyses of the hindquarter vascular bed, in most cases utilizing paired experiments, to compare the resistance responses in hypertensive and matched normotensive control rats.

Three groups of paired animals are utilized for this purpose: 1) 10 young, "prehypertensive" SHR, 4-week-olds (SHR₄), mean arterial pressure (MAP) being 111 ± 1 mmHg as compared with their matched controls (NCR₄) here MAP is 98 ± 1 mmHg. The pressures were measured directly in the cannulated tail artery under awake conditions. 2) 21 adult SHR, (SHR₂₁) with matched controls (NCR₂₁). SHR₂₁ were 6-7 months of age and hence in the "established" phase of hypertension, with an MAP of 150 ± 4 mmHg.

While NCR₂₁ had pressures of 111 ± 3 mmHg. 3) 10 RHR, about 3 months of age at the time of the hemodynamic analysis 6 weeks earlier. Unilateral renal artery clipping had been performed and the subsequent rise in blood pressure is checked 3 weeks later by means of tail artery cannulation and direct MAP measurement. On the day of study MAP was 175 ± 7 mmHg in these RHR as compared with their matched controls (NCR₃) here MAP was 105 ± 2 mmHg.

Dependence on External Calcium for the Noradrenaline Contractility of the Resistance Vessels in Spontaneously Hypertensive and Renal Hypertensive Rats, as Compared with Normotensive Controls

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Abstract

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Isolated hindquarters of rats were perfused at constant flow with a plasma substitute so that pressure responses to various concentrations of noradrenaline (NA) could be measured in consecutive sections of the vascular bed with normal (> 1.5 mM) or low (0.2 mM) Ca^{2+} in the perfusate. The animals used were 6-week-old spontaneously hypertensive rats of the Okamoto strain (SHR_Y), normotensive controls of the same age (NCR_Y), 6-7-month-old SHR (SHR_A) and NCR (NCR_A) and rat made hypertensive by clipping of one renal artery (RIIR) plus matched normotensive controls (NCR_R). Concentration-response curves to NA showed that constrictor responses to NA become more dependent on external calcium as one proceeds peripherally in the vascular system in both SHR_A and NCR_A with responses in proximal end being least and small pre- and postcapillary vessels being most dependent in both cases. In low calcium SHR_A retained their responses better than NCR_A whereas RIIR retained their responses to NA poorer than did NCR_R. No significant differences in responses in low calcium were observed in SHR_Y compared to NCR_Y though a trend in the same direction as in SHR_A could be traced. It is concluded that there is no evidence that altered handling of calcium initiates vascular hyperactivity in SHR but that the handling of vascular calcium in SHR differs from RIIR and both differ from NCR.

The intracellular concentration of calcium ions is of key importance for the excitation-contraction coupling in muscle. However the various types of muscle differ considerably with regard to their intracellular stores of calcium which are utilized in the process of activation. Thus, while in skeletal muscle the amounts of intracellular calcium bound to the sarcoplasmic reticulum are able to account for the total Ca^{2+} release during excitation,

th cardiac and vascular muscles are dependent, to a great extent, on Ca^{2+} influx from the external environment. In this respect, however smooth muscle effectors do vary considerably depending on their location along the vascular bed. For example, at Ca^{2+} concentrations low that the portal vein is completely unresponsive to noradrenaline (NA), aortic strips still retain much of their NA responsiveness. Furthermore, the dependence on Ca^{2+} seems to vary with the type of muscle activation. Thus, upon graded reduction of Ca^{2+} the myogenic activity of the portal vein vanishes well before its responses to NA administration (Sutter 1976), suggesting a more efficient mobilization of calcium stores when membrane depolarization is induced by the adrenergic transmitter than when it occurs "spontaneously".

Recent studies (Folkow *et al.* 1976, Sutter *et al.* 1977) on the responsiveness of the microvascular sections, which are of particular hemodynamic importance because of their control of pre- and postcapillary resistance, capillary flow distribution and of capacitance, indicate a increasing dependence on external calcium the closer one comes to the capillary level. Thus, in this and in several other respects, the smooth muscle of the microvessels is more related to that of the portal vein than is that of the aorta or pulmonary artery while the larger more proximal precapillary vessels appear to fall somewhere in between.

The question arises whether chronic hypertension, besides having hemodynamically important effects on precapillary vessels design in terms of increased media thickness in association with a reduced internal radius (cf Folkow *et al.* 1973, 1974), may also involve changes of smooth muscle function in the microvessels in that the process of excitation-contraction coupling is altered, as reflected, e.g. in a changed dependence on external calcium. In the past few years it has been a subject of much discussion as to whether a change of this coupling might be decisive for the altered reactivity of the resistance vessels (e.g. Fleich and Haensler 1974).

For such reasons, an experimental analysis was performed of the extent to which the resistance responses are affected by Ca^{2+} changes in normotensive Wistar rats (NCR) as compared with young and adult spontaneously hypertensive rats (SHR, Okamoto strain) and renal hypertensive rats (RHR, induced by unilateral renal clipping in NCR). Part of the results has been briefly reported in an earlier paper (Folkow *et al.* 1976).

Methods

1. Material

The present study is based on hemodynamic analyses of the headquarter vascular bed, in most cases utilizing paired experiments to compare the resistance responses in hypertensive and matched normotensive control rats.

Three groups of paired animals were utilized for this purpose. 1) 10 young, "prehypertensive" SHR, 6-week-olds (SHR₆), mean arterial pressure (MAP) being 111 ± 1 mmHg as compared with their matched controls (NCR₆) here MAP = 98 ± 1 mmHg. The pressures were measured directly in the cannulated tail artery under awake conditions. 2) 21 adult SHR (SHR_A) with matched controls (NCR_A). SHR_A are 6-7 months of age and hence in the "established" phase of hypertension, with a MAP of 150 ± 4 mmHg, while NCR_A had pressure of 111 ± 3 mmHg. 3) 10 RHR, about 3 months of age at the time of the hemodynamic analyses. 6 weeks earlier unilateral renal artery clipping had been performed and the subsequent rise in blood pressure as checked 3 weeks later by means of tail artery cannulation and direct MAP measurement. On the day of study MAP was 175 ± 7 mmHg in these RHR as compared with their matched controls (NCR₃) where MAP = 105 ± 2 mmHg.

II Preparation and measurements

R_T measurements. Details of the preparation of the hindquarter vascular beds for paired perfusions, for comparisons of SHR_A – NCR_A , SHR_V – NCR_V and RHR – NCR_R have been described earlier (e.g. Folkow *et al.* 1970; Folkow *et al.* 1974) and only a brief summary will be given below. By occluded mass ligatures the hindquarters of the two animals were completely isolated with the exception of the aorta and the inferior caval vein. The tail and feet were excluded from the circulation, leaving a mass of about 75% of which is made up of skeletal muscle. Mean arterial inflow pressure (P_A) of each preparation was continuously measured by a cannula inserted in the tail artery. Both hindquarters were then perfused in parallel with the same artificial plasma perfusate (4% Dextran in oxygenated Tyrode solution) at constant and equal flows per min and per 100 g of tissue, with the caval vein widely opened so that P_V was *in vivo* zero. In this type of paired experiment the changes in P_A reflect the changes in total resistance to (R_T) in the hindquarter vascular bed under constant flow conditions.

R_{AP} measurements. To estimate possible differences between SHR and NCR with respect to the resistances of proximal parts of the precapillary vessels, more distal pre- and postcapillary resistances, 6 pairs of NCR_A and SHR_A pressure also was measured in a sidebranch to a superficial small artery located on the lower calf (P). As flow was known and kept constant, $P_A - P$ reflects the resistance to flow offered by proximal parts of the pre-capillary resistance vessels (R_{AP}). Some of these measurements were performed on animals where separate R_A and R_V estimations were also made (see below), others in which only R_T was estimated, but both preparations showed closely similar $P_A - P$ responses. Measures of P were initially performed in the intact animals, before the hindquarter preparation was started. The small artery and its cannulated side branch had an external diameter around 0.15–0.20 mm, had not been exposed. Prior to cannulation, it was first completely relaxed by local application of papaverine, but to facilitate the insertion of the cannula and to give its maximal external diameter which was 0.2–0.3 at normal arterial pressure. Its vascular tone was soon regained and then the diameter was again reduced particularly when an increased vasoconstrictor fibre tone was initiated by graded bleeding. — The difference $P_A - P$ also reflects the relative contribution of more “proximal” pre-capillary resistance vessels (R_{AP}) to the total systemic resistance ($R_T = (P_A - P_V)/Q$) because flow is the same along all common vascular sections and P_V can be assumed to be nearly zero.

R_A , R_{AD} and R_V measurements. One group of SHR_A (–6) and NCR_A (–9) were used for separate estimations of the precapillary and postcapillary vessel responses with a technique which has earlier been extensively used for comparing SHR and NCR (cf. Folkow *et al.* 1974). Here the venous outflow pressure (P_V) also was continuously measured in a side branch of the caval vein and P_V could be set at any level by means of an outflow tube inserted in the caval vein. Further the fully isolated hindquarter preparation was continuously weighed with great precision. Since the preparation is far more time-consuming technically demanding, it was not possible to do these particular experiments as paired perfusions of SHR and NCR_A . Instead, SHR_A and NCR_A perfusions were alternately used, great care being taken to perform all preparatory details and manoeuvres in exactly the same way.

Initially the vascular bed was kept maximally dilated by injections of papaverine and P_A and P_V adjusted such that an isogravimetric state was established. Then the capillary filtration coefficient (C) was measured by transiently inducing equal and simultaneous increases of both P_A and P_V . This procedure was followed by inducing selective increases, first of P_A and then of P_V , to such levels that equal degrees of filtration were induced in the two situations. This allows for an estimation of the mean capillary pressure in the isogravimetric state (P_{CI}) (cf. Eliasson *et al.* 1974). Knowing P_A , P_{CI} , P_V and flow it is possible to deduce separately the pre- and postcapillary resistances (R_A , R_V , respectively) in the maximally dilated state.

R_A and R_V could also be estimated when steady state levels of vasoconstriction had been induced: a step increase in the concentration of NA in the infusate (see below), each increase superimposed on the previous pressure level. Here P_O increase, resulting from the postcapillary vasoconstriction in the control flow situation, was reflected by a shift from the isogravimetric equilibrium to increasing degrees of filtration. With correction for concomitant reductions in CFC, as estimated from the volume increase caused by transient P_V increases, it could be deduced how much P_O had increased as a result of the NA constriction. Thus, from the actual levels of P_A , P_O and P_V the respective increases in pre- and postcapillary resistances could, for the purpose of comparison, be deduced with considerable precision by procedures that have been used in several earlier analyses of R_A , R_V , P_O and CFC (cf. Folkow *et al.* 1974; Eliasson *et al.* 1974).

The extent of total resistance responses ($R_T = R_A + R_V$) corresponded closely in these SHR_A – NCR_A used in separate R_A and R_V responses, and in those where only R_T , or R_T together with R_{AP} , was

score 1 was then possible to deduce also the changes of resistance in the "distal" pre-capillary resistance unit (R_{AD}). Flow being known and constant, the changes of $P - P_O$ then reflect the R_{AD} changes, just $P - P$ and $P - P$ reflect the R_{AP} and R changes, respectively.

III. Experimental protocol

In all experiments here P was measured, the $P_A - P$ difference as first studied in the intact SHR and NCR before and after temporary blood losses in order to follow the extent to which the resistance (the proximal pre-capillary vessels (R_{AP})) is involved in neurogenic adjustments in relation to the overall resistance. This as done to see whether the neurogenic R_{AP} responses *in vivo* and the *in vitro* responses to A corresponded as to their magnitude.

When the artificial perfusion was started, the headquarter vascular beds were, in most cases, first perfused in the plasma substrate containing low Ca^{2+} concentration (0.2 mMol/l). This was continued for at least 10 min to ask out local extracellular stores of Ca. At intervals the Ca^{2+} content of the venous effluent was measured and fell rapidly (within 3-10 min) to level that stabilized around 0.3-0.4 mMol. It then slowly rose into the blood stream as going on throughout the low Ca^{2+} perfusion, presumably largely coming from the considerable Ca stores also the large muscle mass and, to some extent, from the retina. Therefore, the immediate Ca^{2+} environment of the vascular smooth muscle at the steady state medium of low Ca^{2+} perfusion may be expected to be closer to 0.3-0.4 mMol than to 0.2 mMol, but was usually equal in the paired preparations since they were identically treated and showed the same cross Ca^{2+} concentrations. Early during the initial period of Ca^{2+} set-out, repeated papaverine injections were referred to secure an initial, fully relaxed state for the vascular beds. It is during this state of complete relaxation that the measurements for estimating P_{Cl} and CPC are performed in the $P_A - P$ system on SHR and NCR.

The NA infusions are then administered into common mixing chamber so that both preparations always received equal NA concentrations and flows. When the vascular response to given NA concentration had reached steady state steps are made in NA infusion as performed, until the maximum tolerable vasoconstrictor (pressure) response had been achieved. This is tested by finally increasing the NA concentration until no further pressure changes could be induced in the constant flow situation. Usually 6-7 different NA concentrations are used in each experiment in order to provide satisfactory dose-response "resistance" curve.

Next the NA infusion is stopped and the vessels allowed to relax fully. Subsequently the NA infusion is again started, usually at the lowest NA concentration that had earlier produced barely noticeable response. When this set response was fully re-established, $CaCl_2$ infusion is added to the mixing chamber to give largely normal Ca^{2+} concentration of the perfusate (1.5 mMol/l or above). As soon as the Ca^{2+} ions reached the tissues there was an immediate and pronounced enhancement of the vasoconstrictor response. This is also the case when higher NA concentrations are tested in this way. The full range of NA concentrations and vasoconstrictor responses were repeated at the approximately normal Ca^{2+} concentration, up to the maximum obtainable constriction (pressure) levels.

Once this had been obtained, the NA infusion is again stopped and the vessels were allowed to fully relax once more. This is secured by means of repeated papaverine injection. Usually the whole procedure, from the first NA infusion to the last at low and high Ca^{2+} levels, respectively took about 2 hr. In some experiments the resistance responses to NA were tested in the reverse order i.e. starting at normal Ca^{2+} levels, but in principle the results were the same, particularly concerning the relationships between the vasoconstrictive and hypertensive animals.

Considerable edema formation occurred only if the intense constrictions at the highest NA infusions at normal Ca^{2+} were allowed to last for longer periods. The reason is that only then are more powerful precapillary constrictions induced. Such, because of the constant flow perfusion, proportionally increases P and hence causes more pronounced filtration. The modest rise in "tissue pressure" that in such situations could be induced towards the end of the experiment could, however be estimated after full vascular relaxation by stopping the inflow and measuring the modest rise in "residual" P_A level. This type of slight disturbance affected only the final few NA infusion episodes at normal Ca^{2+} contraction and could, moreover easily be corrected for.

On the basis of the recorded changes in pressure drop along the vascular bed as whole, and along the consecutive vascular sections, dose-response "resistance" curves could be calculated and plotted, for both the entire vascular bed and for its respective consecutive resistance sections at "low" and at "normal" Ca^{2+} concentrations. For statistical evaluation of the results the pairing design t-test was used in all experiments.

where the paired perfusion technique was utilized. When this was not the case as when R_A and R_I perfusions were performed in SIIR_A and NCR_A the group comparison (t -test) was used. Differences considered to be significant at the $P < 0.05$ level.

Results

1. NA effects on total resistance (R_T) at high and low Ca^{2+}

In paired, constant flow perfusions of the hindquarter vascular beds the relationship between NA concentration and pressure (P_A) responses (reflecting directly the total resistance R_T , responses since flow was constant) was studied at normal (1.5–2 mM) and low mM) Ca^{2+} concentrations in the following groups of animals: 1) SIIR_Y in comparison with age- and sex-matched NCR_Y; 2) SIIR_A and their matched NCR_A; and 3) RIIR (unilateral renal clipping during about 6 weeks) and their NCR_R. The arrangement of paired perfusions greatly facilitated the comparison between the normotensive and hypertensive vascular beds since perfusate composition, flow, temperature, NA and Ca^{2+} concentrations were then identical in both. Consequently the ratio between the responses of the hypertensive and normotensive vascular beds was quite consistent within each group of animals, although variations between experiments were somewhat larger, presumably due to accidental differences in temperature, concentrations, perfusate composition etc. Nevertheless, with occasional exceptions, the spread within each group of paired animals was quite small, particularly considering the fact that the pressure (resistance) responses amplify the underlying changes in vascular smooth muscle length to about the fourth power according to Poiseuille's law.

As normotensive controls, Wistar rats from the same breeding colony were used in all 3 groups, but since they were of different ages they differed moderately in mean arterial pressure (MAP) being in NCR_Y 98 ± 1 mmHg, in NCR_R 105 ± 2 mmHg and in NCR_A 111 ± 3 mmHg. This age-dependent difference in pressure was reflected also in the curves relating NA dose to resistance response ("resistance curves") at normal Ca^{2+} insofar as the maximal pressor responses to NA (MPR) were 167 ± 11 mmHg for NCR_Y, 188 ± 11 mmHg for NCR_R and 213 ± 15 mmHg for NCR_A. MPR, estimating the maximal contractile strength of the media, largely reflects the media thickness in relation to the internal radius, particularly when huge amounts of Ba⁺⁺ are added to ensure that all contractile sites are engaged (cf. Folkow *et al.* 1973, 1974). However, in this particular study only NA was used, but earlier experiences have revealed a fairly constant relationship between maximal NA and maximal Ba⁺⁺ responses in NCR, as well as in SIIR and RIIR.

A close relationship between MAP and MPR (and also resistance at maximal vasodilation and steepness of the resistance curves) was noted also when the hypertensive rats were compared with their respective controls at normal Ca^{2+} , in agreement with earlier findings (cf. Folkow *et al.* 1973, 1974; Lundgren 1974). Thus, in SIIR_Y MAP was 111 ± 1 mmHg with a MPR of 207 ± 11 mmHg (25% higher than in NCR_Y). In SIIR_A these values were 150 ± 4 mmHg and 308 ± 14 mmHg (45% higher than in NCR_A) and in RIIR 175 ± 7 mmHg and 263 ± 2 mmHg (40% higher than in NCR_R). The MPR relationships between the three hypertensive groups and their normotensive controls are also schematically illustrated in Fig. 1A. However, as also observed earlier (Lundgren *et al.* 1974), the relationship between

and MAP was considerably higher in SHR_A (308/150 - 4.05) than in RHR (265/175 - 1.52), indicative of a relatively more pronounced media thickening in SHR_A, as is usually also the case with left ventricular hypertrophy in SHR and RHR, when related to their respective MAP. Concerning other characteristics of the resistance curves such as the NA threshold, ED₅₀, etc., the three NCR groups were closely similar and so also were the hypertensive groups, again in agreement with earlier observations. In such resistance curves ED₅₀ denotes 50% of the maximum reduction in average internal radius of the respective resistance vessels.

At low Ca_e, the various NCR groups also showed a close correspondence concerning the NA resistance curves and their maximal pressor responses. However even though the ratio between the normotensive and hypertensive resistance curves was also quite consistent within each group, a considerable discrepancy was seen at low Ca_e between SHR and NCR, between RHR and NCR and particularly when comparing SHR with RHR. This is illustrated in Fig. 1 where the effects of normal and low Ca_e²⁺ on the R responses to NA are compared in the three paired groups of animals. Section A of this Figure illustrates the maximal R_T responses at normal Ca_e when the NCR value is standardized to 100% in order to more clearly show the relative deviations from the normotensive controls of the maximal R responses in SHR, SHR_A and RHR. The responses at low Ca_e are also indicated in section A by the hatched areas and are expressed as per cent of the respective maxima at normal Ca_e.

In section B of Fig. 1 the maximal NA responses at low Ca_e are again shown for NCR, RHR, SHR and SHR_A. To facilitate a direct comparison between them, all the responses at normal Ca_e are set to 100%, so that the deviations of the SHR, SHR_A and RHR responses at low Ca_e²⁺ from those of NCR are more readily seen.

It is clear from Fig. 1 that both the RHR and SHR responses at low Ca_e deviate substantially from those of NCR, and in opposite directions. The differences from NCR are highly significant for both RHR and SHR_A ($p < 0.01$), but not for SHR though SHR_T show the same tendency as SHR_A, i.e. better maintained NA responses at low Ca_e than in NCR. In other words, the resistance vessels of the renal hypertensive rats and the spontaneously hypertensive rats behaved quite differently at low Ca_e, the former showing a less pronounced NA response than the normotensive controls, the latter a more pronounced one. The difference between RHR and SHR is, of course, even more significant ($p < 0.001$).

11.4 effects on consecutive resistance sections (R_{AT} , R_{AD} , R)

R_{AD} . As already indicated in an earlier study (Sutter *et al.* 1977), the "proximal" pre-arteriolar vessels, as defined also in Methods, appear to contribute towards 25% of the total resistance (R) upon intensified sympathetic discharge in rats. This was judged from the increased $P - P$ difference in relation to $P - P$ during stepwise increases of blood flow. The same is true for SHR, though the absolute R_{AD} increase was here greater and in proportion to the greater $P - P$ ($\sim R_T$) difference in SHR. During "resting equilibrium" *in vivo* on the other hand, R_{AD} contributes only some 10% to R in both NCR and SHR. This indicates that in both the normotensive and hypertensive rats the larger proximal resistance vessels are relatively more involved in the remote neurogenic control of

where the paired perfusion technique was utilized. When this was not the case, as when R_A measurements were performed in SHR_A and NCR_A, the group comparison *t* test was used. Differences considered to be significant at the $P < 0.05$ level.

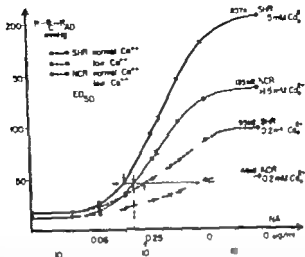
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1) NA effects on total resistance (R_T) at high and low Ca_o

In paired constant flow perfusions of the hindquarter vascular beds the relation between NA concentration and pressure (P_A) responses (reflecting directly the total R_T responses since flow was constant) was studied at normal (1.5–2 mM) and 1 mM Ca_o concentrations in the following groups of animals: 1) SHR_T in comparison with age- and sex-matched NCR_T; 2) SHR_A and their matched NCR_A; and 3) RHR (after renal clipping during about 6 weeks) and their NCR_B. The arrangement of paired perfusions greatly facilitated the comparison between the normotensive and hypertensive vascular beds since perfusate composition, flow, temperature, NA and Ca_o concentrations were then identical in both. Consequently the ratio between the responses of the hypertensive and normotensive vascular beds was quite consistent within each group of animals. Variations between experiments were somewhat larger, presumably due to accidental differences in temperature, concentrations, perfusate composition, etc. Nevertheless, occasional exceptions, the spread within each group of paired animals was quite small, particularly considering the fact that the pressure (resistance) responses amplify the lying changes in vascular smooth muscle length to about the fourth power according to Poiseuille's law.

As normotensive controls, Wistar rats from the same breeding colony were used in 3 groups, but since they were of different ages they differed moderately in mean arterial pressure (MAP), being in NCR_T 98 ± 1 mmHg, in NCR_B 105 ± 2 mmHg and in NCR_A 111 ± 3 mmHg. This age-dependent difference in pressure was reflected also in the relating NA dose to resistance response ("resistance curves") at normal Ca_o . Thus, the maximal pressor responses to NA (MPR) were 167 ± 11 mmHg for NCR_T, 171 ± 11 mmHg for NCR_B and 213 ± 15 mmHg for NCR_A. MPR, estimating the maximal contractile strength of the media, largely reflects the media thickness in relation to the internal diameter, particularly when huge amounts of BaCl₂ are added to ensure that all contractile sites are engaged (cf Folkow *et al.* 1973, 1974). However, in this particular study only NA was used, but earlier experiences have revealed a fairly constant relationship between maximal NA and maximal BaCl₂ responses in NCR, as well as in SHR and RHR.

A close relationship between MAP and MPR (and also resistance at maximal vasodilation and steepness of the resistance curves) was noted also when the hypertensive rats were compared with their respective controls at normal Ca_o . In agreement with earlier findings (cf Folkow *et al.* 1973, 1974; Lundgren 1974). Thus, in SHR_T MAP was 111 ± 1 mmHg with a MPR of 207 ± 11 mmHg (25% higher than in NCR_T). In SHR_A these values were 150 ± 4 mmHg and 308 ± 14 mmHg (45% higher than in NCR_A) and in RHR 175 ± 7 mmHg and 265 ± 22 mmHg (40% higher than in NCR_B). The MPR relationships between the hypertensive groups and their normotensive controls are also schematically illustrated in Fig. 1A. However, as also observed earlier (Lundgren *et al.* 1974) the ratio between



3 Dose-response R_{AD} curves to NA for SHR_A and NCR_A bloodquarter vascular beds at normal and low Ca_o during constant flow perfusion. The arrows indicate points (s) the curves where prior responses are maintained in magnitude and therefore allow comparison of the NA concentrations required to produce identical constrictions of the distal, precapillary resistance vessels at normal and low Ca_o in SHR_A and NCR_A. ED_{50} here denotes the point where 50% of the maximum reduction in average internal radius of the distal resistance vessels is reached.

the R_{AD} response remains in both NCR_A and SHR_A. Both ways of expressing the responses are justified and, in other words, the proximal resistance vessels behave quite similarly in both groups of animals.

R_{AD} . The maximal NA responses of the distal precapillary resistance vessels in SHR_A and NCR_A are shown in the middle part of Fig. 2, which also illustrates the extent to which these responses are reduced at low Ca_o . Since this vascular compartment, corresponding to the small distal arterioles and metarterioles, provides the dominating contribution to R_T and is, moreover, primarily responsible for the important myogenic tone and for local adjustments of flow and its microvascular distribution, the complete curves relating NA doses to precursor (resistance) responses for the R_{AD} section are also illustrated, both at normal and at low Ca_o (Fig. 3). As mentioned in Methods, these R_{AD} "resistance curves" were computed by subtracting R_{AP} and R from R_T even though all the various measurements could for technical reasons only exceptionally be performed in the same experiment. However, there was such a close correlation with regard to the magnitude of the R_T responses to NA in expts. where either P_A alone, P_A and P_{AO} , or P_{AO} , P_{AP} , P_{AO} , and F were estimated, particularly regarding percentage differences in SHR_A/NCR_A relationships, that the results could be pooled. Furthermore, the R_{AD} and R responses differed little between expts. and constituted such relatively small fractions of R_T during vasoconstriction, that possible errors in measurement will have little effect only on the computation of R_{AD} .

Figures 2 and 3 illustrate how the R_{AD} responses were normally much steeper in SHR_A and also better maintained at low Ca_o than in NCR_A. The resistance curves shown in Fig. 3 reveal that this was not only at maximal NA values, but also throughout the whole

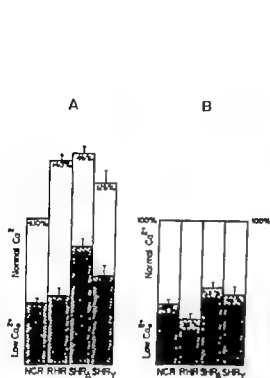


Fig. 1

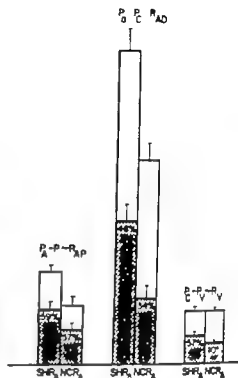


Fig. 2

Fig. 1. Section A compares the effect of normal and low Ca_0 on the R_T responses to NA in NCR, RHR, SHR_A and SHR_V. To facilitate the comparison the maximum R_T response at normal Ca_0 is NCR expressed as 100%. All other values are expressed as percentage of NCR control values, with each individual group being compared to its own matched control group.

Section B compares the same maximal R_T responses to NA at normal and low Ca_0 in NCR, RHR, SHR_A and SHR_V but now with each R_T at normal Ca_0 expressed as 100%. This allows for a more direct comparison of the extent of reduction at low Ca_0 of the maximum R_T response to NA in NCR, RHR, SHR_A and SHR_V. Note that both RHR and SHR_A differ significantly from NCR but in opposite directions.

Fig. 2. Shows the maximum resistance responses to NA in consecutive sections of the hindquarter recorded at normal and low Ca_0 . Results are expressed as mmHg pressure with hatched areas representing responses at low Ca_0 expressed as a percentage of those at normal Ca_0 . R_{AP} denotes resistance to flow in proximal precapillary (arterial) resistance vessels, R_{AD} that in distal precapillary resistance vessels and R_V that in the postcapillary (venous) resistance vessels.

resistance than in the local myogenic tone, the latter being predominantly a function of the distal precapillary resistance vessels.

During constant flow perfusion and NA infusion, R_{AP} also increased approximately to the same extent as during enhanced vasoconstrictor fibre discharge. Thus, at maximal NA vasoconstriction the proximal precapillary resistance vessels contributed 19 and 21% respectively of the total resistance in NCR_A and SHR_A (cf. Fig. 5). At low Ca_0 the responses of the proximal precapillary resistance vessels were essentially equally reduced in NCR_A and SHR_A. When expressed in terms of the maximal $P_A - P_V$ difference, achieved during supramaximal NA infusion during low Ca_0 , 57 and 59% of the $P_A - P_V$ response at normal Ca_0 remained as illustrated in Fig. 2. If one instead considers the R_{AP} response in terms of the $P_A - P_V$ difference after subtracting the $P_A - P_V$ difference at complete vascular relaxation (thus expressing the extent of smooth muscle shortening) almost exactly 50

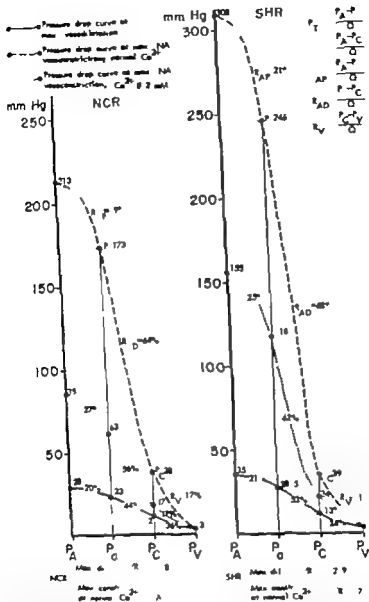


Fig. 5 A diagrammatic representation is shown of the pressure drop along the consecutive vascular segments of the perfused hindquarters in NCR and SHR during 1) complete vascular relaxation, 2) maximal NA concentrations at low calcium and 3) maximal NA concentrations at normal calcium.

of resistance vessels (R_T in comparison with R_{AP} , R_{AD} and R_V) to induce the same degree of vasoconstriction as that produced by $7.5 \cdot 10^{-4}$ mol/l of NA (0.15 g/ml) on the NCR vessels at normal Ca_0 . The Figure expresses in another way what has been stated above, i.e. that while there was no significant difference between NCR and SHR as to the main-

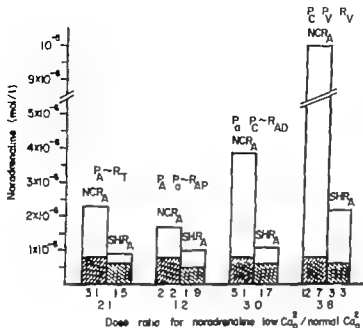


Fig. 4. Concentrations of a noradrenaline which are equivalent to the concentration of 7.5×10^{-8} mol/l ($0.15 \mu\text{g}$) in NCR at normal Ca_v^{2+} . Consecutive vascular sections NCR_A and SHR_A at low Ca_v^{2+} (open columns) and at normal Ca_v^{2+} (hatched columns) are compared.

range of NA concentrations. This fact will of course, greatly increase confidence concerning differences observed than if only the effect of one NA concentration had been tested. This also holds true for all the other comparisons discussed above. This Figure also allows a comparison of the NA concentration needed to produce identical pressor (resistance) responses in SHR_A and NCR_A both at normal and low Ca_v (cf Fig. 4). From the model NCR_A response to $0.15 \mu\text{g}/\text{ml}$ of NA at normal Ca_v (dotted line), it can be seen that the NA concentration must be increased relatively much more in NCR_A (from 0.15 to $0.8 \mu\text{g}/\text{ml}$, i.e. some 5-fold) than in SHR_A (from 0.12 to $0.20 \mu\text{g}/\text{ml}$, i.e. about 2-fold) to produce the same pressor response at low Ca_v^{2+} (see arrows). Thus, when compared in this way it is also clear that the distal pre-capillary vessels maintained their contractility to NA much better in SHR_A than in NCR_A at low Ca_v .

R_V The right part of Fig. 2 shows the maximal venous resistance responses to NA at normal and low Ca_v in SHR_A and NCR_A. In contrast to the precapillary resistance vessels the venous resistance vessels did not differ significantly at normal Ca_v but, again, the SHR response was better maintained than the NCR_A one at low Ca_v . This was the case whether one, as here, depicts the highest $P_0 - P_V$ difference achieved at maximal NA concentration or if one first subtracts from this value the $P_0 - P_V$ difference present at complete vascular relaxation. The former reflects the maximal contractile strength of the venous resistance vessels, the latter rather reflects their degree of shortening, but confronted with a relatively constant transmural pressure thanks to the constant flow. Both ways of describing the venous responses have their justification and both reveal a less well maintained response in NCR_A compared with SHR_A at low Ca_v .

III Integrated R_T , R_{AP} , R_{AD} and R_V responses in SHR_A and NCR_A

In Fig. 4 a comparison is made of the difference between SHR_A and NCR_A with respect to how much the NA concentration has to be increased for the various consecutive sections

ormally responsible for myogenic tone and are also an important part of neurogenic tone, seem to be almost entirely dependent on Ca_0 for smooth muscle activation in the Osborne rat (Sotter *et al* 1977). Therefore, these important microvessels show a closer sensitivity to the smooth muscle of the portal vein than to that of the aorta. The present study shows that resistance vessels of SHR and RHR in this respect differ but in opposite directions, from the resistance vessels of the normotensive controls with regard to their response on Ca_0 for contractility to the adrenergic transmitter NA. Thus, the SHR vessels exhibited better maintained NA responses at low Ca_0 than the NCR vessels but the difference was only clearly obvious in the well-established phase of hypertension in SHR₁₆; there was only a tendency in this direction in SHR. These results are in general agreement with recent findings *in vitro* on subcellular fractions of SHR mesenteric arteries, which indicate a better calcium storing capacity in SHR vascular smooth muscle (Wei, Janis and Daniel 1976). The most reasonable explanation of the present findings concerning the resistance vessels in SHR is that they have an increased amount of calcium available to contractile machinery. In RHR, on the other hand, the NA responses of resistance vessels were more depressed in low Ca_0 than those of the NCR, as though the smooth muscle cells per unit mass retain or possess smaller calcium stores in RHR than in NCR. It is of interest that the resistance vessels of the genetically hypertensive rats of the New Zealand strain (OHR) in this respect seem to be more related to the RHR vessels than to the SHR ones, to judge from findings on the GHR mesenteric vessels (Jones, Lyon and Imai 1973).

At normal levels of external Ca_0 both SHR and RHR show the same type of vascular reactivity and it is consistent with altered wall to lumen ratios. For these reasons the cellular hyper-reactivity of both RHR and SHR, as revealed in the perfusion study, seems to be entirely a matter of structural precapillary adaptation of the vascular bed (cf Folkow *et al* 1973). Furthermore, the fact that the difference between SHR and NCR concerning Ca_0 dependence paralleled, rather than preceded, genetically-linked primary hypertension strongly suggests that this changed Ca_0 dependence reflects any primary trigger element in terms of an altered excitation-contraction coupling preceding the hypertensive state. In this respect, there is a clear contrast, as to the enhanced central nervous "hyperactivity" in SHR, which is particularly marked in the early "prehypertensive" stage, and the neuroendocrine links then brings about an enhanced excitatory drive on the SHR cardiovascular system (Hallböök and Folkow 1974).

Nevertheless, it is clear that an alteration of the cellular handling of Ca^{++} has, indeed, taken place in SHR₁₆, consistent with an increase in available calcium, either intracellular or membrane-bound, and that the situation in this respect is quite different in RHR and that they differ quantitatively from NCR. The simplest explanation of the present findings in RHR is that the vascular smooth muscle effectors, along with their functional, chemical and structural adaptation in response to the enhanced neurohormonal excitatory drive, have developed an improved capacity for the extracellular stores to hold calcium, consistent with the findings of Wei, Janis and Daniel (1976). Tentatively this could be due to development of the sarcoplasmic reticulum to an extent that more than matches the increased formation of contractile filaments that occurs in connection with smooth muscle hyper-

tenance of the NA response in the R_{AP} section at low Ca_o^{++} the responses in the R_A , R_V sections were much better maintained in SHR_A than in NCR_A at low Ca_o and thus consequently also the case for R_T (cf Folkow *et al* 1976). The difference appears particularly marked for the venous resistance vessels, which was due to the fact that NCR_A dose-response curve was markedly displaced to the right at low Ca_o^{++} .

In Fig. 5 the present results on SHR_A and NCR_A are schematically summarized in a different way *i.e.* as the profile of pressure drop along the hindquarter vascular bed, at complete vascular relaxation and at maximal NA vasoconstriction at either a high (>1.5 mM) or low (0.2 mM) Ca_o^{++} . This way of illustration perhaps gives the best impression of the marked differences between NCR_A and SHR_A in terms of their peripheral hemodynamics, which ensue from the different design of their precapillary vessels, where those of SHR_A are characterized by narrowed lumina and a relatively thicker media, *i.e.* an low w/r_1 . Besides having a considerable influence already during maximal dilatation, this a vascular architecture further implies a marked precapillary "hyperreactivity" during increasing vasoconstriction in SHR_A and the end-result of this is shown in the Figure: at maximal NA vasoconstriction at normal Ca_o . Fig. 5 also illustrates the relatively greater dependence on Ca_o of the NCR_A resistance vessels compared with the SHR_A ones by the difference of the pressure drop curves at low Ca_o , but where the vessels were again exposed to normal NA concentrations. These curves are based on the "weighed" mean values from a number of SHR_A - NCR_A comparisons performed in the present study and in general these results closely resemble those of an earlier analysis of the pre- and postcapillary resistance response in SHR and NCR hindquarters performed on animals of about the same age (Folkow 1974).

Discussion

The present experiments were aimed at exploring some aspects of excitation-contraction coupling in the systemic resistance vessels by comparing the dependence on Ca_o^{++} of the responsiveness to noradrenaline (NA) of the hindquarter vascular beds of normotensive (NCR), renal hypertensive (RHR) and spontaneously hypertensive rats (SHR). The question was whether these types of secondary and genetically linked, primary hypertension, differently differ mutually and in relation to normotensive controls, as to vascular contraction to NA when the Ca_o^{++} concentration is drastically lowered.

It is well-known, as outlined in Introduction, that various types of muscle can differ markedly in terms of their intrinsic Ca_o stores and dependence of Ca_o and this is the case even when different parts of the systemic vascular bed are compared, such as aortic portal vein smooth muscle (Sutter 1976). Furthermore it is much debated whether an alteration in the intracellular handling of calcium, and hence of the excitation-contraction process, might be one of the basic phenomena behind the changed control of the enhanced resistance. If for example, such an altered calcium handling were one of the initiating mechanisms behind the enhanced resistance, and, furthermore, was common to all types of hypertension it might already be expected to reveal itself in the early phases of hypertension and exhibit similar signs of alteration independent of how the hypertensive state was initiated. It has recently been shown that the resistance vessels closest to the capillary level, w

creased stores of intracellular Ca^{2+} . Neither is there any *a priori* reason to imagine all mechanisms on the cellular-subcellular level behind these two different types of extension.

It, by no means, denies that the excitation-contraction coupling remains an indispensable in the whole process of cardiac and vascular contraction, but from this it does not follow that "primary" changes in this coupling process should necessarily constitute a starting process, either in SHR or RHR (or GHR) hypertension. At least the present findings lend no support to such a view though they do indicate that changes in the contraction coupling must have taken place both in SHR and RHR. These changes may well be of a dietary nature and reflect different ways of effector cell adaptation to the new situation.

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trophy. Such enhanced cellular stores of calcium are, however, evidently also present in the postcapillary smooth muscles, since SHR also differs here from NCR, despite the fact that the venous side hardly seems to be exposed to any increased pressure and does not show any clear signs of either true media hypertrophy or any hyper-reactivity concerning the resistance responses. For such reasons also, the suggested enhancement of the intracellular calcium storage is *not* a likely explanation for the resistance vessel hyperreactivity, inasmuch as this seems to be confined to the precapillary resistance vessels only (cf Folkow 1974) at least in the hindquarters. It should be noted, however, that Simon (1976) reported altered capacitance function in SHR, but this may partly represent other vessel sections than the postcapillary resistance ones, and their characteristics are not necessarily identical. Further, the present findings show that also the proximal precapillary resistance vessels displayed hyperreactivity, but they did not differ from those of the NCR as to Ca_0 .

Thus, it seems logical to assume that the characteristic pre-capillary hyperreactivity in SHR is mainly due to the structurally increased wall/lumen ratio of these vessels, more so as the same type of hyper-reactivity is seen in the similarly altered RHR vessels. In the RHR vessels the intracellular stores of calcium appear, as mentioned, to be relatively reduced compared with normotensive controls, possibly because the increased form of contractile filaments within the muscle cells is here not matched with a corresponding increase of sarcoplasmic stores of calcium. Why the RHR (and evidently also GHR, as mentioned above) smooth muscle cells differ from the SHR ones in this latter respect is not known. *A priori* it might reflect either a genetically-linked difference in smooth muscle behaviour and adaptation to the increased activation and load. It is also possible that the different types of excitatory influences in SHR and RHR (and GHR) imply differences concerning so-called "trophic" influences, that seem to modify the structural adaptation of the cardiovascular effector cells when exposed to the high pressure state (cf Folkow 1975).

Whatever the case, it seems, at least from the point of view of the present findings, that the same mechanisms of altered intracellular handling of calcium are not crucial for the initiation and maintenance of both the SHR and the RHR hypertension. If altered handling of calcium is involved in RHR, it is manifest only at normal levels of external calcium. Both types of hypertension may so far be adequately explained by the combined influence of the above-mentioned extrinsic excitatory influences on the cardiovascular effector cells and their rapidly developed "structural autoregulation" which tends to form a positive feedback interaction with such influences (cf Folkow 1975). Possibly the relatively low intracellular storage capacity of the RHR vessels smooth muscle might imply difficulties for these cells to rapidly enough eliminate the increased influx of Ca_0 upon excitation, which might help to prolong and enhance their contractile response, other things being equal. After all, our earlier study in NCR (Sutter *et al.* 1977) suggests that most of the energy needed for the contraction of the true resistance vessels has to be derived from the extracellular environment of the cells, and then they are faced with the time problem of getting rid of intracellular Ca^{2+} concentration rapidly enough to allow for relaxation. The same tentative explanation cannot possibly be used also in SHR, which behave as if their blood vessels

Methods

Surgery procedures. The experiments were performed on 12 cats, weighing 2.2–3.8 kg, fasted for 4 h. Animals were anaesthetized with chloralose (30 mg/kg) after induction with ether. A tracheal cannula was inserted to allow free airway. The abdomen was opened in the midline and the greater splanchnic, the aorta and the spleen were extirpated.

Animals were heparinized (3 mg/kg). A femoral artery was cannulated and connected to a mercury manometer for continuous recording of arterial blood pressure.

Blood flow recordings. To record the magnitude of the overall colonic blood flow, side-bore polyethylene tubes were inserted into the superior mesenteric vein and the venous outflow was diverted to a peristaltic drop chamber filled with silicone oil, and returned to the animal via the external jugular vein. A drop counter operated as ordinary star recording on smoked kymograph paper; the height of the meniscus being inversely proportional to the rate of the blood flow (see figures). For technical reasons the *in situ* blood flow in some animals had to be determined by arterial inflow measurement. For this purpose a polyethylene tube was inserted in the left carotid artery and the blood flow was diverted into the recorder unit, as here connected to the proximal end of the centrally cut superior mesenteric artery. In some experiments the inferior mesenteric artery was also severed, a procedure that has been demonstrated to alter total colonic blood flow in the cat (Heldin 1969). Irrespective of the method used the regional blood flow was qualitatively and quantitatively the same, and by using arterial inflow recording close intraluminal infusion of drugs (see below) was greatly facilitated. In all experiments the peritumoral vasoconstrictor fibres along the superior and inferior mesenteric artery were dissected free and severed.

Mechanical stimulation. Mechanical mucosal stimulation was performed by pulling slightly wetted swabs by rotating movements through the colonic lumen. The swabs were kept small enough not to cause distension of the colon. All the "stimulator" was inserted into the lumen either through small incision in the proximal end or at the midpoint of the colon. The latter method was chosen when the effects of mechanical stimulation of the proximal and distal half of the colon were to be studied separately. In some animals small latex balloons were also used for studying the effects of distension. For this purpose the colon was carefully inflated with air to pressure of about 20 mmHg.

Nerve stimulation. In all but 4 experiments the pelvic nerves were dissected free on both sides and divided so they emerged from the sacral roots. The distal ends were mounted on silver-ring electrodes for constant stimulation. In 6 experiments the distal cut ends of the peritumoral vasoconstrictor fibres along the superior and inferior mesenteric arteries were also mounted on electrodes for studying the effects of mechanical stimulation during sympathetic nervous stimulation. Supramaximal square wave pulses were delivered from Grass Stimulator (model S 5 E) to the pelvic nerves (1 V, 5 ms) and the sympathetic bundles (1 V, 3 ms). Generally 4–10 Hz were used, which corresponds to the upper range in the physiological charge rates.

Administration of drugs. Atropine sulphate was administered in doses of 1 mg/kg. Clove oil, administration of 5-HT (5-hydroxytryptamine, 5HTA, Chemical Co.) and dihydroergotamine (DHE, Maasbroek, Lundol) into the superior mesenteric artery were either (venous outflow recordings) performed through cannula inserted into superior mesenteric artery or (arterial inflow recordings) via the polyethylene tube between the drop recorder and the superior mesenteric artery. 5-HT was administered either as bolus injection or as continuous infusion with an infusion pump. DHE, blocking the effects of 5-HT was given as bolus injection in the superior mesenteric artery while occluding the supplying artery. When testing the occlusion venous blood was collected for 1–2 min to reduce the amount of DHE reaching the mesal circulation. In order to obtain blockade of the adrenergic α_1 - and α_2 -receptors, phenoxybenzamine (Dienylac, Senik, Kiers & French Lab Ltd.) 5 mg/kg and propranolol (Indinal[®], ICI-Pharma) 3 mg/kg were given. The blocking effects were tested by means of sympathetic nerve stimulation (α_1 -blockade) and isoprenaline (β -blockade).

Results

1 The effect on colonic blood flow of mechanical stimulation of the mucosa

With the pelvic nerves bilaterally severed mechanical mucosal stimulation of the proximal colon either by means of friction stimuli or distension elicited a marked blood flow increase amounting to about 90 per cent of control flow (Fig. 1 left panel). The blood flow increase,

Vascular Responses to Mechanical Stimulation of the Mucosa of the Cat Colon

By

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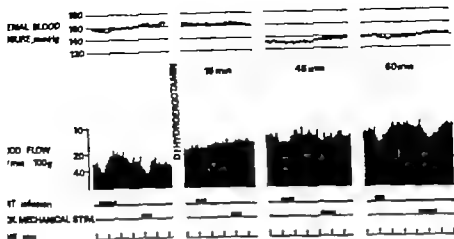
Abstract

FASTH, S., L. HULTÉN, O. LUNDGREN and S. NORDGREN: *Vascular responses to mechanical stimulation of the mucosa of the cat colon*. Acta physiol. scand. 1977 101 98-104.

Mechanical stimulation of the mucosa of the proximal colon evoked hyperemia which also could be elicited when severing the autonomic fibres to the colon. Mechanical stimulation of the distal colon evoked hyperemia which, on the other hand, was abolished by cutting the pelvic nerves. Afferent nerve stimulation evoked a response similar to that seen when stimulating the distal colon. Adrenergic and cholinergic receptor blockers did not affect the studied colonic vasodilatations. Dihydroergotamine given in doses abolishing the vascular effects of L.A. injected 5-hydroxytryptamine abolished the hyperemia to mechanical stimulation of the proximal colon but not that of the pelvic stimulation. It is concluded that a local nervous vasodilator reflex exists in the proximal colon similar to that earlier demonstrated in the small bowel (Biber *et al.* 1971) and that the reflex vasodilatation evoked by mechanical stimulation of the distal colon is mediated via the pelvic nerves. Hence, the vascular control differs in the proximal and distal parts of the colon.

Recent studies on vasodilator mechanisms in the small intestine of the cat have provided evidence that mechanical stimulation of the jejunal mucosa induces a substantial transintestinal vasodilatation which is probably transmitted via an intrinsic nervous reflex dependent on the release of 5-hydroxytryptamine (5-HT) (Biber, Lundgren and Svaz 1971; Biber, Fara and Lundgren 1974). Furthermore, this vascular response is neither blocked by cholinergic or adrenergic blocking agents, nor by ganglionic blocking agents (Biber *et al.* 1971). It has also previously been shown that mechanical stimulation of the rectal mucosa gives rise to a pronounced transient vasodilatation in the colon (Hultén 1969), but that the sacrospinal reflex response, which is associated with a colonic contraction, is dependent on intact pelvic nerves. Moreover, both the motor and the vasodilator responses are atropine resistant and the transmitter mechanisms involved are unknown.

The aim of the present study was to explore if a local vasodilator response similar to that in the small intestine could be elicited by mechanical stimulation also of the colonic mucosa and to study the role of 5-HT in the reflex vasodilatation associated with mechanical stimulation of the rectal mucosa.



2. Cat 38 kg. The vasodilatation induced by mechanical mesocol stimulation and intravenously infused 5-HT before and at varying intervals after giving DHE. Note that the vascular responses which are selectively blocked after 15 min, gradually return to reach control level 60 min after DHE administration.

The effects of a pharmacological 5-HT blockade on the studied vasodilator responses

ie vasodilatations induced by mechanical stimulation and pelvic nerve stimulation were abolished before and after giving dihydroergotamine (DHE). To test the effect of this blockade, HT was injected or infused into the colonic circulation before and after DHE administration. Administration of 5-HT either as a bolus injection (10 μ g) or infusion (50–100 μ g/min), evoked a vasodilatation of the same type as that produced by mechanical mucosal stimulation. As is seen in Fig. 2, DHE (1 μ g/kg) administered as a close i.a. injection during systemic circulatory arrest, completely abolished this vasomotor effect.

During such a 5-HT blockade mechanical stimulation failed to produce any vasoconstriction (Fig. 2). However, the short-lasting vasoconstriction previously described as an initial effect of mechanical stimulation remained and was in fact sometimes prolonged, outlasting the period of stimulation (see panel C of Fig. 3).

The 5-HT blockade remained efficient for some 40 min but after that period the vasodilatation elicited by stimulation of the proximal colonic mucosa exhibited the same magnitude as before the blockade. In this situation the vasomotor effects of 5-HT could also be reversed (Fig. 2). The vasodilatation evoked by pelvic nerve stimulation was not abolished, however (Fig. 3) and there was still a sustained colonic contraction, indicating that both the vasodilator and motor responses on pelvic nerve stimulation were unaffected by the 5-HT blockade.

¹ The effects of mechanical stimulation during activation of sympathetic nerves and during α - and β -adrenergic blockade

When mechanical stimulation of the proximal colonic mucosa was performed during continuous vasoconstrictor fiber stimulation the same vascular response as during control conditions was observed though of a lower magnitude. The peak reduction of the vascular

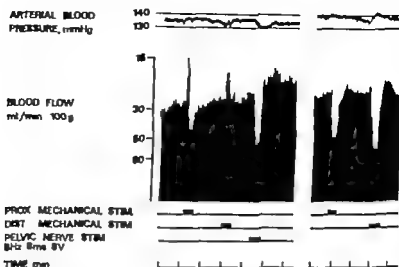


Fig. 1 *Left panel.* Cat 2.3 kg (severed pelvic nerves). The effect of proximal and distal mucosal stimulation as compared to that of efferent pelvic nerve stimulation on colonic blood flow. Note that the blood increase could only be elicited on mechanical stimulation of the proximal colon.

Right panel. Cat 3.1 kg (intact pelvic nerves). The effect of proximal and distal mucosal stimulation on colonic blood flow. Note that distal stimulation now evokes a blood flow increase, indicating a reflex through the pelvic nerves.

often preceded by a transient decrease, reached its maximum within 30 s after commencement of the stimulation and returned to control level 1–3 min following its cessation. The blood flow response could be reproduced throughout the experiment. When the mucosa of the resting proximal colon was just pinched without any stretching of the mesentery or distension of the colonic wall, the same flow response was evoked. In contrast, neither mechanical nor electrical stimulation of the distal colon caused any increases in the blood flow, although sometimes a shortlasting decrease of blood flow was seen (Fig. 1 left panel).

Efferent pelvic nerve stimulation induced the earlier reported pronounced colonic dilatation (Fig. 1 left panel). When comparing this blood flow response with the one elicited by mucosal stimulation a difference was observed. Although there was a pronounced blood flow increase occurring within some 5–10 s after commencement of the nervous stimulus, reaching its maximum within 20–30 s, this blood flow increase vanished within 30–40 s and there was also a sustained motor contraction of the colon. This was never observed on proximal mechanical mucosal stimulation or distension, although an augmented rhythmic peristalsis was occasionally observed.

In experiments with the pelvic nerves intact, mechanical stimulation of the proximal colonic mucosa gave rise to a blood flow increase which was similar to that observed when the pelvic nerves were severed. Mechanical stimulation of the distal colonic mucosa produced a blood flow increase which was in some experiments more marked than that caused by proximal stimulation (Fig. 1 right panel). The shortlasting blood flow decrease which was often observed on proximal mucosal stimulation was also observed on distal mucosal stimulation. Simultaneously with the vanishing blood flow increase, occurring within 30–40 s, there was also a sustained motor contraction, similar to that seen on pelvic nerve stimulation.

mucosa of the proximal colon is mediated by a local nervous reflex as in the small bowel (Per *et al* 1971, 1974). The vasodilatation observed upon stimulating the distal colon was essentially the same as the hyperemic response earlier reported by Hultén (1969). A shortlasting vasoconstriction was often observed just before the hyperemic response on mucosal stimulation. A similar response pattern was reported by Biber *et al.* (1971) on mechanically stimulating the small bowel mucosa. These authors ascribed this effect a stimulation artefact caused by the handling of the intestinal segment before stimulating mucosa. In the present study however the initial vasoconstriction appeared even when touching the mucosa which could not possibly exert a mechanical interference with the blood outflow. Neither α - and β -blocking agents, nor DHE had any influence on this vasoconstriction which seems to be caused by some unknown mechanism.

Dihydroergotamin (DHE), in doses blocking the effect of LA injected 5-HT was the only pharmacological agent tested in this study which could abolish the vasodilatation evoked by stimulating the mucosa of the proximal colon. The intestinal wall contains large amounts of 5-HT which is mainly located in the enterochromaffin cells (Viall 1966, Penttilä and Samuelson 1968) and also, according to some authors, in some of the nerve cells that constitute the intramural plexa (Gershon and Ross 1968). The physiological significance of the 5-HT stores is largely unknown (Furness and Costa 1973). In view of the present findings and earlier reports on the effects of mechanical stimulation on jejunal blood flow in the cat (Biber *et al* 1971, 1974, Biber 1973), it seems possible that 5-HT released by even slight stimulation of intestinal mucosa, plays an important role in the functional hyperemia of the gut at least in the small bowel and the proximal colon. From a theoretical point of view it appears attractive to assume that local factors are involved in the control of a functional mucosal hyperemia producing localized blood flow increases to meet with increased nutritional demands in different parts of the intestine during the digestive work.

While the results of the present study seem to indicate that 5-HT is probably involved in the functional hyperemia of the proximal colonic mucosa, this is apparently not the case in the distal colonic part, where another vasodilator mechanism is operating. As has been previously demonstrated (Hultén 1969) this part of colon is controlled by the pelvic parasympathetic nerves. Activation of these nerves gives rise to a marked mucosal hyperemia, associated with an increased secretion and a tonic motor contraction. The motor and vascular responses are atropine-resistant and direct and indirect evidence favours the presence of a humoral-like mechanism (Faith and Hultén 1973, Faith 1973, Faith, Hultén, Johnson and Zetlin 1976).

Earlier studies have shown that nervous effects exerted on the musculature and on the vascular bed are differently organized in the proximal and distal half of the colon and that this differentiation might have important functional implications (Hultén 1969). Thus the proximal part of the colon exhibits a characteristic motility pattern which favours absorption, whereas the motility pattern seen in the distal part would rather favour storage and expulsion. Moreover the proximal part differs from the distal part insofar that it is devoid of centrally controlled vasodilator fibres. In many respects therefore the proximal colon is functionally very similar to the small intestine, and the results of the present study lend further support to the view that the colon is comprised of two functionally different units.

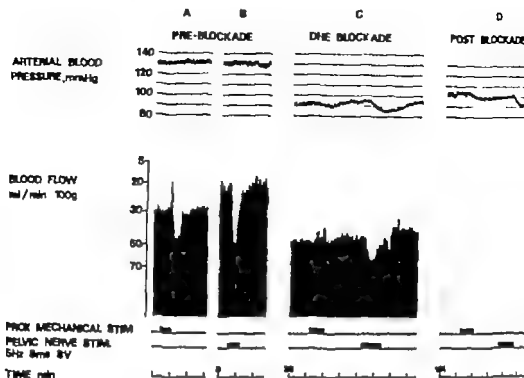


Fig. 3 Cat 2.3 kg. The effect of DHE-blockade on the colonic blood flow responses to proximal mechanical stimulation and to efferent pelvic nerve stimulation. Panels A and B show the responses before the block. Panel C shows the effects during DHE-blockade. Note that the blood flow increase evoked by mechanical stimulation of the proximal colon is completely abolished during the DHE-blockade, whereas the response of pelvic nerve stimulation is virtually unchanged. Panel D shows the returning response of proximal mucosal stimulation about 1 h after DHE was given.

resistance amounted to about 20 per cent compared to a maximal reduction of about 50 per cent during prestimulatory control. Neither propranolol (3 mg/kg i.v.) nor phentylephrine (5 mg/kg i.v.) affected the vascular response to mechanical stimulation.

Discussion

The present study has demonstrated that stimulation of the mucosa of the proximal part of the acutely denervated colon produced a marked hyperemia while no vasodilatation was seen when stimulating the distal part of the denervated colon. In the innervated large bowel vasodilatations were evoked by mechanical stimulation in the proximal as well as in the distal parts of the colon. The hyperemia seen in the proximal colon exhibited all the characteristic features earlier reported by Biber *et al.* (1971, 1974) with regard to the vasodilatation evoked by mechanical stimulation of the small intestinal mucosa. Hence, the vasodilatation preceded by a shortlasting vasoconstriction, could be induced in the denervated colon. Furthermore, the hyperemia was not blocked by adrenergic or cholinergic receptor blocking agents. However, it could be abolished by administration of a 5-HT receptor blocking agent. Although the effect of a nerve conductivity blocking substance such as e.g. tetrodotoxin was not tested in this study it is proposed that the vasodilatation observed when stimulating

the increase of the proximal colon is mediated by a local nervous reflex as in the small bowel (Biber *et al.* 1971, 1974). The vasodilatation observed upon stimulating the distal colon was apparently the same as the hyperemic response earlier reported by Hultén (1969).

A shortlasting vasoconstriction was often observed just before the hyperemic response upon mucosal stimulation. A similar response pattern was reported by Biber *et al.* (1971) when mechanically stimulating the small bowel mucosa. These authors ascribed this effect to a stimulation artefact caused by the handling of the intestinal segment before stimulating the mucosa. In the present study however the initial vasoconstriction appeared even when flushing the mucosa which could not possibly exert a mechanical interference with the venous outflow. Neither α - and β -blocking agents, nor DHE had any influence on this vasoconstriction which seems to be caused by some unknown mechanism.

Dihydroergotamin (DHE), in doses blocking the effect of L.A. injected 5-HT was the only pharmacological agent tested in this study which could abolish the vasodilatation evoked by stimulating the mucosa of the proximal colon. The intestinal wall contains large amounts of 5-HT which is mainly located in the enterochromaffine cells (Vialli 1966, Penttilä and Lempiäinen 1968) and also, according to some authors, in some of the nerve cells that constitute the intramural plexa (Gershon and Ross 1968). The physiological significance of these 5-HT stores is largely unknown (Furness and Costa 1973). In view of the present findings and earlier reports on the effects of mechanical stimulation on jejunal blood flow in the cat (Biber *et al.* 1971, 1974, Biber 1973), it seems possible that 5-HT released by even slight stimulation of intestinal mucosa, plays an important role in the functional hyperemia of the gut at least in the small bowel and the proximal colon. From a theoretical point of view it appears attractive to assume that local factors are involved in the control of a functional mucosal hyperemia producing localized blood flow increases to meet with increased nutritional demands in different parts of the intestine during the digestive work.

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Reflex Plasma Hyperglycemia and Hyperosmolality Evoked by Unloading of the Carotid Baroreceptors

By

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Abstract

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Hemorrhage usually accompanied by considerable increase in the plasma osmolality and glucose concentration due to an augmented release of glucose from the liver. In the present experiments an attempt is made to investigate the possible role of different vascular receptors in mediating this hyperglycemic (hyperosmolar) response. Bilateral vagotomy or stimulation of the carotid chemoreceptors by perfusion of the carotid sinus with venous blood at normal pressure only slightly increased the arterial plasma glucose concentration. On the other hand, when the sinus nerves were cut in the vagotomized animal, thereby simulating complete unloading of the carotid baroreceptors, the arterial plasma glucose concentration rose by about 8 mM/L and the arterial plasma osmolality by about 10 mOsm/kg H₂O. Perfusion of the carotid baroreceptors with arterial blood at different levels of hypotension showed that the baroreceptor-induced hyperglycemia is graded in relation to the pressure level. Regional hypotension of the liver, pancreas, skeleton, kidneys or brain did not significantly affect plasma glucose concentration or osmolality. We conclude that the reflex release of glucose from the liver during hemorrhage mainly is mediated from the unloading of arterial baroreceptors.

It has been known for some years that hemorrhage is associated with a pronounced increase in the plasma osmolality (*e.g.* Bergentz and Brief 1965, Baue *et al.* 1967, Boyd and Mansberger 1968). Recent studies from this laboratory have demonstrated that the hyperosmolality during acute hemorrhagic hypovolemia can be mainly attributed to hyperglycemia (*cf.* Bernard 1877, Robertson 1935, Brooks *et al.* 1963) and, further, that it contributes in an important way to the plasma volume restoration by causing a glucose osmotic transcapillary absorption of fluid from the extravascular compartment into the blood stream (*cf.* Järehult *et al.* 1972, Järehult 1973, 1975 b, Järehult *et al.* 1976). The hyperglycemia also seems to expand the overall extracellular fluid volume in bleeding by producing glucose-osmotic fluid withdrawal from the intracellular to the interstitial space (Järehult 1975 b). The hyperglycemia is established by an increased release of glucose from the liver (*cf.* Schenk 1894) and seems to be mediated by several different links of the sympatho-adrenal system, i.e. by a direct adrenergic influence on the liver cells and,

indirectly by adrenergic control of catecholamine and glucagon release from the *adn* and pancreas (Järhult 1975 a)

The direct adrenergic effects on the heart and the blood vessels in hemorrhage are *lv* to be initiated from several receptor areas in the circulatory system (*e.g.* Heymans 1958 Öberg 1964 Chlen 1967), but the receptors mediating the metabolic *lv* glycemic response to bleeding have so far not been identified. An attempt was *ma* the present investigation to analyse the possible role of certain cardiovascular *recepto* this hyperglycemic response to hemorrhage.

Methods

30 cats (mean b.wt. 3.2 kg) were used in the study. The animals fasted 12–14 h before the experiment received water *ad libitum*. They were anesthetized *lv* with chloralose (50 mg/kg) and urethane (30 mg/kg) after induction with ether. A tracheal cannula was inserted and the animal breathed spontaneously throughout the experiment. Heparin (1 000 IU/kg b.wt.) was given prior to intravascular cannulation. brachial artery was cannulated for recording systemic arterial pressure and for sampling of arterial *lv*. Heart rate was recorded with a Grass tachograph (triggered by the systolic pressure wave). Muscle *lv* flow was estimated in some of the experiments by measuring with a optical drop recorder the *outflow* blood flow from the cannulated right femoral vein. In these expts. the right paw was removed. external pressure applied to the thigh so that flow through venous vessels other than the *carotid* femoral vein was prevented without interfering with the arterial inflow.

In an attempt to reveal the possible role of various receptors in the *lv* circulatory system in the *lv* glycemic (hyperosmolar) response to hemorrhage, arterial plasma glucose concentration and *osmo* were followed in the normotensive animal during alterations of receptor activity resembling *quasi* those occurring in bleeding. For this purpose, the carotid chemoreceptors were stimulated and the *lv* baroreceptors unloaded in cat after bilateral cervical vagotomy. The effects of vagotomy *per se* on studied. The possible influence on plasma glucose concentration and osmolality from portalated *in* and receptor sites in the renal, splanchnic and cerebral circulations was investigated as well (see *Discussion*).

The carotid chemoreceptors were stimulated according to the technique described by Little and *C* (1975). The right iliac artery and vein were cannulated with polyethylene tubings and the arterial *lv* venous flows diverted through two separate channels of Harvard peristaltic pump. Distal to pump arterial and venous tubings joined to a common tubing which after bifurcation was connected to the common carotid arteries. All main arterial vessels in the vicinity of the carotid body were then *lv* except for the external carotid arteries which were cannulated and the outflows diverted to a fused *lv* right external jugular vein. The carotid intra sinus pressure was recorded bilaterally via T-tubes in external carotid arteries and could be controlled by means of screw clamps placed distal to the T-tube. With this technique for artificial flow perfusion, the chemoreceptors in the isolated carotid sinus-*gl* regions could be stimulated by switching flow from arterial to venous blood perfusion under *circumstan* constant, normal pressure in the carotid sinus. The normotension was accomplished by adjustments on peristaltic pump and the screw clamps.

The carotid baroreceptors were inactivated in vagotomized cat with two different techniques. (1) A *lv* complete unloading of the baroreceptors was stimulated by cutting the sinus nerves bilaterally in the other intact sinus regions. (2) The above described preparation for artificial perfusion of the carotid *lv* sinus was used for graded inactivation of the baroreceptors. For this purpose, the sinus regions were *perf* with arterial blood at mean inflow pressures of about 120, 90, and 60 mmHg, respectively.

Local splanchnic hypotension was produced with adjustable screw clamp placed on the coeliac superior mesenteric artery. Regional blood pressure was recorded in the left gastric artery or in a jejunal artery. Local renal hypotension was accomplished by a screw clamp placed on shunt *lv* connecting the right iliac artery and the renal arteries. Renal inflow pressure was measured from T-tube the shunt distal to the clamp.

Regional cerebral hypotension was accomplished in the following ways: A In experiments, *st* connected the left iliac artery and both vertebral arteries, which were dissected free and cannulated above the thoracic aperture. Another shunt connected the right iliac artery and both common car

TABLE 1. Effects on mean systemic arterial blood pressure (AP), heart rate (HR), arterial plasma osmolality and arterial plasma glucose concentration evoked by interference with cardiovascular receptors and by regional (aortic) hypotension. Reported changes refer to maximal effects in individual experiments and to the interindividual range.

pericardial resection	Increase of AP (mmHg)	Increase of HR (beats/min)	Increase of osmolality (mOsm/kg H ₂ O)	Increase of glucose (mM/L)
bilateral vagotomy	5	unchanged	unchanged	1-4
removal of carotid baroreceptors	5	5-20	0-10	1-5
partial unloading of aortic baroreceptors over hypotension 30 mmHg	9	30-135	20-110	8-14
distal hypotension 30 mmHg	3	unchanged	unchanged	unchanged
proximal hypotension 30 mmHg	3	unchanged	unchanged	unchanged
distal hypotension 90 mmHg	3	unchanged	unchanged	unchanged
proximal hypotension 90 mmHg	4	unchanged	unchanged	unchanged

arteries. The main nerves were cut and about 10 mm later the vertebral and carotid blood pressure was allowed to fall to 30-60 mmHg by adjusting screw clamps placed on each artery. In 2 expts. only the carotid clamp was made, leaving the carotid arteries and other nerves left intact. Cerebral hypotension to 30 mmHg was achieved by adjustments of the screw clamp on the arterial aorta.

Hemorrhagic hypotension was accomplished in some experiments by bleeding the animal into a graduated pressure bottle connected via T-tube to the right brachial artery. The exsanguination was kept so that the arterial blood pressure reached the level of 30 mmHg within 3 min, this pressure level was then maintained during 30 min. At the end of the pressure bottle.

Blood pressure, heart rate and blood flow were recorded on a Grass Polygraph,atham F13 AC transducers were used for the pressure measurements. Arterial plasma osmolality was determined with the osmometer (Osmometer 31 L.A.S. Advanced Instruments, Inc.) and arterial plasma glucose concentration with the glucose-oxidase technique.

In Results section data are given as mean values \pm S.E.

Results

In the control period before receptor interference, mean arterial blood pressure (AP) averaged 130 \pm 5 mmHg, heart rate (HR) 185 \pm 4 beats/min, arterial plasma osmolality 330 \pm 2 mOsm/kg H₂O and arterial plasma glucose concentration 10.3 \pm 0.7 mM/L.

A survey of the effects caused by interference with the activity of different receptors on AP, HR, and on arterial plasma glucose concentration and osmolality is given in Table 1. The results are described in some detail below.

Effects evoked from the carotid chemo- and baroreceptors

Bilateral cervical vagotomy was made about 30 min before interference with the carotid receptor activity. The vagotomy *per se* leading to elimination of the afferent discharge from the aortic, cardiac and pulmonary mechanoreceptors, increased the arterial plasma glucose concentration and osmolality slightly whereas AP and HR, except for an initial transient decrease, remained essentially constant.

Indirectly by adrenergic control of catecholamine and glucagon release from the adrenal and pancreas (Järhult 1975 a)

The direct adrenergic effects on the heart and the blood vessels in hemorrhage are known to be initiated from several receptor areas in the circulatory system (e.g. Heym, Neil 1958, Öberg 1964, Chien 1967) but the receptors mediating the metabolic hypoglycemic response to bleeding have so far not been identified. An attempt was made in the present investigation to analyse the possible role of certain cardiovascular receptors in this hyperglycemic response to hemorrhage.

Methods

30 cats (mean b.wt. 3.2 kg) were used in the study. The animals fasted 12-14 h before the experiment and received water *ad libitum*. They were anesthetized i.v. with chloralose (50 mg/kg) and urethane (0.5 mg/kg) after induction with ether. A tracheal cannula was inserted and the animal breathed spontaneously throughout the experiment. Heparin (1 000 IU/kg b.wt.) was given prior to intravascular cannulation. The brachial artery was cannulated for recording systemic arterial pressure and for sampling of arterial blood. Heart rate was recorded with a Grass tachograph triggered by the systolic pressure wave. Mean blood flow was estimated in some of the experiments by measuring with an optical drop recorder the outflow of blood from the cannulated right femoral vein. In these experiments, the right paw was removed and external pressure applied to the thigh so that flow through venous vessels other than the cannulated femoral vein was prevented without interfering with the arterial inflow.

In an attempt to reveal the possible role of various receptors in the circulatory system in the hyperglycemic (hyperosmolar) response to hemorrhage, arterial plasma glucose concentration and osmolality were followed in the normotensive animal during alterations of receptor activity resembling qualitatively those occurring in bleeding. For this purpose, the carotid chemoreceptors were stimulated and the carotid baroreceptors unloaded in cats after bilateral cervical vagotomy. The effects of vagotomy *per se* were also studied. The possible influence of plasma glucose concentration and osmolality from postulated median receptor sites in the renal, splanchnic and cerebral circulations was investigated as well (see Discussion).

The carotid chemoreceptors were stimulated according to the technique described by Little and Öberg (1975). The right iliac artery and vein were cannulated with polyethylene tubings and the arterial and venous flows diverted through two separate channels of Harvard peristaltic pump. Distal to peristaltic arterial and venous tubings joined to a common tubing which after bifurcation was connected to the two common carotid arteries. All main arterial vessels in the vicinity of the carotid body were then ligated except the external carotid arteries which were cannulated and the outflows diverted to a second right external jugular vein. The carotid intra-arterial pressure was recorded bilaterally via T-tubes in the external carotid arteries and could be controlled by means of screw clamps placed distal to the T-tubes. With this technique for arterial flow perfusion, the chemoreceptors in the isolated carotid sinus-plasma regions could be stimulated by switching from arterial to venous blood perfusion under circumstances of constant, normal pressure in the carotid sinus. The normotension was accomplished by adjustments of the peristaltic pump and the screw clamps.

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Local splanchnic hypotension was produced with an adjustable screw clamp placed on the coeliac superior mesenteric artery. Regional blood pressure was recorded in the left gastric artery or in a small jejunal artery. Local renal hypotension was accomplished by a screw clamp placed on a short branch connecting the right iliac artery and the renal arteries. Renal inflow pressure was measured from a T-tube in the shunt distal to the clamp.

Regional cerebral hypotension was accomplished in the following ways. In 2 experiments, the shunt connected the left iliac artery and both cerebral arteries, which were dissected free and cannulated just above the thoracic aperture. Another shunt connected the right iliac artery and both common carotid

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30 mmHg	3	unchanged	unchanged	unchanged	unchanged
arterial hypotension 30 mmHg	3	unchanged	unchanged	unchanged	unchanged
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Blood pressure, heart rate and blood flow are recorded on Grass Polygraph. Statikon P23 AC transducers are used for the pressure measurements. Arterial plasma osmolality was determined with the osmotic cryoscopy (Osmometer 31 LAB, Advanced Instruments, Inc.) and arterial plasma glucose concentration with the glucose-oxidase technique.

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30 cats (mean b wt. 3.2 kg) were used in the study. The animals fasted 12-14 h before the experim received water *ad libitum*. They were anesthetized i with chloralose (50 mg/kg) and urethane (mg/kg) after induction with ether. A tracheal ca. nula was inserted and the animal breathed spontaneously throughout the experiment. Heparin (1 000 IU/kg b wt.) was given prior to intravascular cannulation. brachial artery was cannulated for recording systemic arterial pressure and for sampling of arterial blood. Heart rate was recorded with a Gram tachograph triggered by the systolic pressure wave. Muscle blood flow was estimated in some of the experiments by measuring with a optical drop recorder the out flow from the cannulated right femoral vein. In these expts. the right paw was removed. external pressure applied to the thigh so that flow through venous vessels other than the can femoral vein was prevented without interfering with the arterial inflow.

In an attempt to reveal the possible role of various receptors in the circulatory system in the glycemic (hyperosmolar) response to hemorrhage, arterial plasma glucose concentration and osm were f lowed in the normotensive animal during alterations of receptor activity resembling qualit those occurring in bleeding. For this purpose, the carotid chemoreceptors were stimulated and the baroreceptors unloaded in cats after bilateral cervical vagotomy. The effects of vagotomy *per se* t studied. The possible influence on plasma glucose concentration and osmolality from postulated rec receptor sites in the renal, splanchnic and cerebral circulations was investigated as well (see Diets

The carotid chemoreceptors were stimulated according to the technique described by Little and (1975). The right iliac artery and vein were cannulated with polyethylene tubing and the arteri venous flows diverted through two separate channels of Harvard peristaltic pump. Distal to post arterial and venous tubings joined to a common tubing which after bifurcation was connected i t common carotid arteries. All mai rterial vessels in the vicinity of the carotid body were then i except for the external carotid arteries which were can nulated and the outflows diverted to a funnel right external j gular vein. The carotid (intra-a) ual pressure was recorded bilaterally via T-tubes external carotid arteries and co ld be co ntrolled by means of screw clamps placed distal to the T. With this technique for artificial flow perfusion, the chemoreceptors in the isolated carotid sinu regions could be stimulated by switching from arterial t venous blood perfusion under circumscri constant, normal pressure in the carotid si us. The normotension was accomplished by adjustments peristaltic pump and the screw clamps.

The carotid baroreceptors were inactivated in vagotomized cat with two d fferent techniques. (1) A plete unloading of the baroreceptors was simulated by cutting the sinus nerves bilaterally in the ock j tact sinus regions. (2) The above described preparation for rificial perfusion of the carotid sinus r was used fo graded inactivation of the baroreceptors. For this purpose, the sinus regions were pc with arterial blood at mea inflow pressures f bout 120, 90, and 60 mmHg, respectively.

Local splanchnic hypotension was produced with an adjustable screw clamp placed on the coel superior mesenteric artery. Regional blood pressure was recorded the left gastric rtery or in a jejunal artery. Local renal hypotensio was accomplished by a screw clamp placed on a shunt i connecting the right iliac artery and the renal arteries. Renal inflow pressure was measured from a T-t the shunt distal to the clamp.

Regional cerebral hypotension was accomplished in the following ways. A i 2 experiments, connected the left iliac artery and both vertebral arteries, which were dissected free nd cannulate above the thoracic aperture. Another shunt connected the right iliac rtery and both common i

TABLE II Effects on mean systolic arterial blood pressure (AP), heart rate (HR), arterial plasma glucose concentration, and arterial plasma osmolality evoked by stepwise reduction of the isolated carotid sinus pressure from about 120 mmHg down to 90 and 60 mmHg, respectively. The animals were vagotomized in the neck about 30 min before experimental intervention. Data are expressed as the mean values from 3 experiments

	Carotid sinus pressure ~ 120 mmHg	Carotid sinus pressure ~ 90 mmHg			Carotid sinus pressure ~ 60 mmHg		
		3 min	6 min	10 min	3 min	6 min	10 min
(mmHg)	124	176	155	147	185	138	118
(beats/min)	198	220	217	221	231	233	235
glucose (mM/L)	12.8	14.6	15.5	15.5	14.8	18.5	18.9
osmolality (mOsm/kg H ₂ O)	321	324	325	324	327	328	330

vascular resistance in the lower leg. Arterial plasma osmolality also rose promptly when it reached level about 9 mOsm/kg H₂O above control and this level was then roughly sustained during the rest of the observation period. The arterial glucose concentration creased rapidly to a steady level about 8 mM/L above control and thus accounted for the major part of the hyperosmolar response.

In 3 expts., the carotid baroreceptors were unloaded by stepwise reduction of the mean arterial pressure from a control level of about 120 mmHg down to 90 and then to 60 mmHg. Table II shows that AP, HR, arterial plasma glucose concentration and osmolality changed clearly and in a graded manner in response to these different levels of baroreceptor activity.

30 min after the sinus nerves were cut in the above described experiments (Fig. 1), the animals were rapidly tied to an AP level of 50 mmHg maintained for another 30 min. On an average, 35% of the blood volume was shed after 30 min of hypotension and at that time there had occurred further increase of the plasma hyperosmolality by about 12 mOsm/kg H₂O and of glucose concentration by about 9 mM/L. This increment of the osmolality and glucose concentration can partly be explained by the decrease in blood volume (see Discussion), but the finding might also suggest that receptors other than those lying in the aortic and sinus nerves are involved in the hyperglycemic (hyperosmolar) response to hemorrhage. The possibility was approached in the experiments described below.

Effects of regional hypotension

Splanchnic and renal hypotension. Lowering of the arterial inflow pressure to 50 mmHg in the liver and intestine, or in the kidneys, did not cause any significant changes of the arterial plasma osmolality or glucose concentration, nor did it affect AP, HR or lower leg blood flow.

Cerebral hypotension. Reduction of the cerebral arterial inflow pressure to 50–60 mmHg with the two different techniques outlined in Methods did not cause any significant changes of AP, HR, plasma osmolality or glucose concentration.

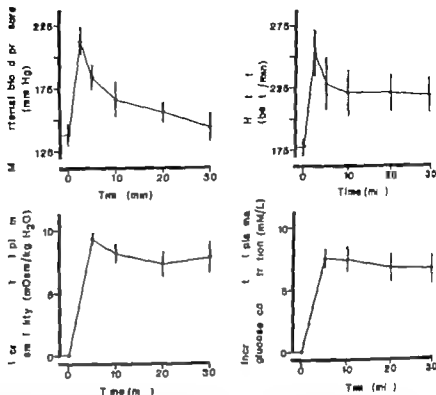


Fig. 1 Changes of mean arterial blood pressure, heart rate, arterial plasma osmolality and arterial plasma glucose concentration in response to bilateral cutting of the sinus nerves. The animals were vagotomized in the neck about 30 min before the sinus nerve section. Mean values \pm S.E. are given.

Stimulation of chemoreceptors These receptors were stimulated by shifting from arterial to venous blood perfusion of the sinus-glomus regions, maintaining the intra-sinusual pressure at a normal level. The induced decrease in pH and pO₂ and the increase in pCO₂ during venous perfusion will here determine the extent of receptor activation. During arterial perfusion pO₂ averaged 10.6 kPa, pCO₂ 3.8 kPa, and pH 7.38. During venous perfusion the corresponding values were 5.2 kPa, 6.1 kPa and 7.24 which are figures similar to those occurring during severe hypovolemia (e.g. Schweitzer and Howland 1972). Such chemoreceptor stimulation caused a moderate increase in AP as well as in skeletal muscle vascular resistance, whereas HR remained unchanged or increased slightly (in accordance with Little and Öberg 1975). A small but invariable increase was seen in the arterial plasma glucose concentration and osmolality.

Unloading of baroreceptors A selective and complete elimination of the discharge from the carotid baroreceptors, without introducing an artificial perfusion system, was achieved by sectioning of the sinus nerves in the vagotomized cat. Fig. 1 shows the changes of AP and HR and the increase of arterial plasma osmolality and glucose concentration during an observation period of 30 min after section of the sinus nerves. HR increased markedly to attain a steady level about 50 beats/min above control. AP rapidly reached a peak increase about 80 mmHg above control, after which it gradually declined towards the control level. The changes of HR and AP were associated with a clear-cut increase of the

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Discussion

The present study shows that unloading of the carotid baroreceptors evokes a marked increase in the arterial plasma glucose concentration with an attendant increase in arterial plasma osmolality. Preliminary experiments indicate that such a response is evoked also from the aortic baroreceptor region. The carotid and aortic baroreceptors can initiate quite a drastic "metabolic" adjustment besides the important reflex effect on the heart and the blood vessels.

Numerous studies have demonstrated that hemorrhage is associated with hyperglycemia (for ref. see Järhult 1975 b). This investigation seems to indicate that the hemorrhagic hyperglycemia to a large extent is initiated from unloading of the carotid arterial baroreceptors, but the quantitative importance of these receptors in the response is difficult to evaluate exactly from the present data. A direct comparison between the magnitude of hyperglycemia evoked by complete elimination of the baroreceptor discharge in normovolemic animals and that seen after bleeding to 50 mmHg (see Results and Järhult *et al.* 1976) suggests that about 50 per cent of the latter response would be due to the baroreceptor reflex. It should be pointed out, however, that the plasma concentration of course increases much more during hypovolemia than during normovolemia, simply because of the much smaller plasma volume available for glucose dilution in the former situation. Furthermore, the marked decrease of blood flow through most tissues during hemorrhage implies that a significantly lower capillary transfer of glucose from the intra- to the extravascular space will occur during bleeding than during baroreceptor inactivation in the resting normovolemic animal. These considerations indicate that the baroreceptor reflex is the main mediator of the hemorrhagic hyperglycemia response. It is quite possible, however, that other receptor stations also contribute to this metabolic adjustment. The present results thus suggest that the carotid chemoreceptors are of some importance, while baroreceptors in the renal circulation as well as postulated baroreceptors in the splanchnic (e.g. Tuttle and McCleary 1975) and cerebral (e.g. Hume 1974) circulation do not contribute to the initiation of hemorrhagic hyperglycemia.

The efferent pathways in the hemorrhagic hyperglycemia response involve several components of the sympatho-adrenal system, viz. direct sympathetic fibres to the liver releasing catecholamines from the adrenal glands, adrenergic release of glucagon from the pancreas (Järhult 1975 a) and adrenergic inhibition of the insulin output (Carey *et al.* 1970, Holst *et al.* 1973). It seems likely that the reflex baroreceptor hyperglycemia makes use of these adrenergic mechanisms, an assumption supported by some preliminary experiments which demonstrate that elimination of the carotid baroreceptor discharge leads to a marked increase in the glucagon and decrease in the insulin concentration (Järhult and Holst 1975). In the CNS, the ventromedial part of the hypothalamus may possibly serve to integrate the reflex, since stimulation of this structure has been shown to cause hyperglycemia and increased plasma levels of adrenaline, noradrenaline and glucagon (Dunér 1953, Folbergom and Euler 1954, Frohman and Bernardis 1971).

ing membrane of the cat) and by Gibson and Pollock (1973) anococcygeus muscle of rat). In both cases, the potassium induced contractures were reduced not only by enceptor blockade but also by denervation.

In the present experiments studied in more detail the influence of intramural noradrenaline on potassium induced contracture in the uterus and in the portal vein of the rat. These organs have different adrenergic responses, noradrenaline has a relaxant effect on the uterus (Schild 1967) whereas it activates the rat portal vein (Johansson *et al.* 1967).

Our preliminary results suggested that the potassium induced release of noradrenaline depended on an estrogenic influence on the smooth muscle. This point is further analyzed here.

Material and Methods

Uterine muscle and portal vein of 60 virgin Wistar rats weighing 130–200 g were used. All rats oophorectomized. 30 of them were given 0.5 mg of oestradiol phosphate i.m. immediately after oophorectomy. This produces an almost constant estrogen effect for several weeks in oestrous mice — Decafolway (1954). These rats were killed on day 4 after operation. The other 30 animals did not receive oestrogen treatment and were killed on day 7 after oophorectomy.

Animals from each group were given an i.p. injection of reserpine, 5 mg/kg, 30 h before the expt. In rats, this treatment depletes the stores of catecholamines within 24–36 h (Owman and Ljörberg 1967). **Uterus and mesentery** The uterus was removed and placed in Krebs solution (composition, see below). The tissues of the uterine horns were split open by longitudinal section at the mesenteric border in order. Strips of the uterus, about 15 mm long, were mounted vertically in a 30 ml jacketed organ bath containing Krebs solution. The preparations were actually subjected to tension of 10 mN. Within 10 min, the preparations relaxed to tension level of 1–2 mN which was maintained during the expt. In a stabilizing period of 30 min, during which spontaneous activity developed, the bathing fluid was replaced by an isotonic potassium solution (composition, see below).

The portal vein was exposed and removed together with some surrounding tissue, placed in Krebs fluid, and then dissected free. Ligatures were put around the vein at the point where joins the superior mesenteric vein and at the hepatic end, and the lumen was opened longitudinally. The preparations were tied vertically as described for the uterine strips. Initial tension was adjusted to approximately 0.5 mN, which the length of the vein between the ligatures was about 30 mm. After 30 min, the bathing fluid was replaced by the isotonic potassium solution. In some expts., repeated contractures were produced. The effect of exposure to the potassium-rich solution and also the recovery period (in Krebs solution) between contractures were kept constant at 15 and 30 min, respectively.

The contractile responses were recorded isometrically by tension transducer (Grass FT-03-C) connected to polygraph ink-writer (Grass P7 or Beckman R611).

Measurement of responses Results obtained in expts. on the rat uterus are principally demonstrated as typical records, whereas results from expts. on the rat portal vein are given as average responses. For the latter purpose, the maximum amplitude of each individual preparation during potassium contracture was set at 100 and served as reference. During the first 3 min of contracture, the tension was measured at 0.5-min intervals and expressed as per cent of the maximum of the response. Normalized values from different preparations were pooled, and mean values are calculated at each respective time.

The results showing effects of repeated stimulation are given as the integrated contractile activity during the first 10 min of the contracture. The contraction record was copied on photographic paper and the area under the myogram was cut out and weighed. The first response in series of consecutive contractures was usually the largest and was chosen as the reference and set at 100%. All other responses in the same expt. were expressed in per cent of this maximum contraction.

Statistical significance was determined by Student's *t*-test.

Solutions The muscle strips were bathed in Krebs solution of the following composition (mM): NaCl 119, KCl 4.6, NaHCO_3 20, CaCl_2 1.5, $\text{N}_2\text{H}_4\text{PO}_4$ 1.2, MgCl_2 1.2, glucose 11, pH 7.4. The contracture solution had the following composition (mM): KCl 127, NaHCO_3 20, CaCl_2 1.5, NaH_2PO_4 1.2, MgCl_2 1.2, glucose 11, pH 7.4. The solutions were prepared on the day of the expt. Double-distilled water and analytical grade chemicals were used.

The Role of Intramural Noradrenaline in the Potassium Induced Contracture of Non Estrogenized Smooth Muscle

By

BENGT BENGTSSON

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Abstract

BENGTSSON B. *The role of intramural noradrenaline in the potassium induced contracture of non-estrogenized smooth muscle* Acta physiol scand. 1977 101 112-121

The influence of intramural noradrenaline on potassium induced contractures was studied in preparations of the uterus and the portal vein of the rat. Uterine strips of oophorectomized rats responded with contraction followed by transient relaxation when immersed in a medium containing 127 mM KCl . Reserpinization or blockade of β -adrenoceptors with propranolol greatly diminished transient relaxation. In the isolated portal vein both noradrenaline depletion with reserpine and α -adrenoceptor blockade (phentolamine) reduced the active tension produced in response to the isotonic potassium solution. These results suggest that intramural noradrenaline plays a significant role in the development of contractures evoked by potassium in the uterus and the portal vein of the rat. In the uterus, the released noradrenaline counteracts the development of active tension, whereas in the portal vein, noradrenaline has a contractile effect which is added to that of potassium. Estrogen treatment reduced (portal vein) the contractile effects of intramural noradrenaline. The adrenergic effect of estrogen is possibly due to reduction in the release of noradrenaline from the sympathetic nerve-endings.

Potassium in high concentrations depolarizes the cell membrane and initiates smooth muscle contraction. However it has also been shown that in high concentration it causes a release of noradrenaline from adrenergic nerve-endings (Kirpekar and Wakade 1968), and it is reasonable to presume that released noradrenaline should modify the potassium induced contractures in adrenergically innervated smooth muscle (Furchgott 1955). In support of this idea, several reports describe an inhibitory effect of α -adrenoceptor blocking agents on potassium contractures in vascular smooth muscle (Bevan *et al* 1963 Shibata and Carrier 1967 Somlyo and Somlyo 1969 Somlyo *et al* 1969 Pelper *et al* 1971 Golenhofen *et al* 1973 Massingham 1973). However these effects have not generally been attributed to inhibition of released noradrenaline, but rather to a direct inhibitory effect on the membrane permeability to activating cations (Bevan *et al* 1963 Shibata and Carrier 1967 Somlyo and Somlyo 1969 Somlyo *et al* 1969).

More substantial evidence in favour of the idea that potassium stimulates smooth muscle indirectly through release of noradrenaline has been produced by Cervoni (19

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Material and Methods

Uterine muscle and portal vein of 60 virgin Wistar rats weighing 150–200 g were used. All rats oophorectomized. 29 of them were given 0.5 mg of oestradiol phosphate i.m. immediately after surgery. (This produces an almost constant estrogenic effect for several weeks as reported by Decavalier 1956). These rats were killed on day 4 after operation. The other 31 animals did not receive any treatment and were killed on day 7 after oophorectomy.

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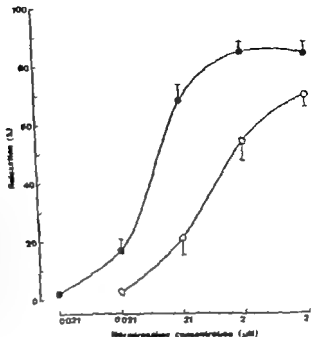
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Potassium in high concentrations depolarizes the cell membrane and initiates smooth muscle contraction. However, it has also been shown that in high concentration it causes a release of noradrenaline from adrenergic nerve-endings (Kirpekar and Wakade 1968) and it is reasonable to presume that released noradrenaline should modify the potassium induced contractures in adrenergically innervated smooth muscle (Furchtgott 1955). In support of this idea, several reports describe an inhibitory effect of α -adrenoceptor blocking agents on potassium contractures in vascular smooth muscle (Bevan *et al.* 1963; Shibata and Cammer 1967; Somlyo and Somlyo 1969; Somlyo *et al.* 1969; Pelzer *et al.* 1971; Golenhofen *et al.* 1973; Massingham 1973). However, these effects have not generally been attributed to inhibition of released noradrenaline but rather to a direct inhibitory effect on the cell membrane permeability to activating cations (Bevan *et al.* 1963; Shibata and Cammer 1967; Somlyo and Somlyo 1969; Somlyo *et al.* 1969).

More substantial evidence in favour of the idea that potassium stimulates smooth muscle indirectly through release of noradrenaline has been produced by Cervoni (1967).



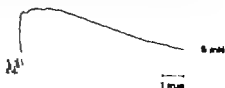
is relevant effect of reserpine on the calcium-induced contracture of the rat uterus, as measured by 3 mM calcium in potassium solution, as a percentage of contraction. Solid circles: Control; Open circles: estrogenized. Each point represents the \pm S.E. of 6 experiments.

b) or addition of 2×10^{-6} M propranolol (Fig. 2 c) or 10^{-6} M atropine to the bathing medium (Fig. 2 d) had no detectable effects on the potassium-induced contracture when animals had been estrogenized. The spontaneous contractile activity was sparse and irregular in the myometrium of estrogenized rats and did not change appreciably after ionization or addition of propranolol.

Effect of reserpine in the depolarized uterus. Fig. 3 shows the relevant effect of reserpine added to a calcium-induced contracture of the depolarized rat uterus. In the estrogenized tissue, maximum relevant effect was attained at approximately $2 \mu\text{M}$ reserpine. Estrogen treatment caused a shift of the log concentration-response curve to the right, increasing the EC_{50} value by about 100 times.

Uterine vein

Depolarized rats without estrogen substitution. Fig. 4 illustrates a typical contractile response of an isolated strip of rat portal vein during immersion in isotonic potassium solution. The tension immediately rises to about maximum, is maintained for about 1 min, then declines slowly.



4 Contractile response of rat portal vein (non-estrogenized) induced by immersion in isotonic potassium solution.



Fig. 1 Spontaneous contractions followed by potassium induced contractions of isolated rat uterus: control, (b) after reserpization, (c) in the presence of 2×10^{-6} M propranolol, (d) in the presence of PGE_1 (6×10^{-6} M).

The bathing solution was kept at 37°C and aerated with a mixture of 5% CO_2 and 95% O_2 .

Drugs: Atropine (BDH), noradrenaline bitartrate (Apoteksbolaget, Sweden), phentolamine (Kary Ciba), polyestradiol phosphate (Estradurin® LEO), propranolol (Inderal ICI Pharma), prostaglandin E_1 (Astra) and reserpine (Serpasil® Ciba) were used in the experiments.

The drugs were injected into the bath in volumes of 0.03–0.1 ml, 10 min before changing the bathing solution to the potassium-rich medium.

Results

I Uterus

A Oophorectomized rats without estrogen substitution The rat uterus responds with contraction when depolarized by potassium. Fig. 1 a shows a typical potassium contracture in a uterine strip from an oophorectomized rat. As can be seen, there is an initial contraction followed by a transient relaxation and a slow rise in tension.

Reserpization greatly diminished the transient relaxation that normally occurs during the first few minutes in a potassium rich solution (Fig. 1 b). The preceding spontaneous contractions were not obviously different after reserpization.

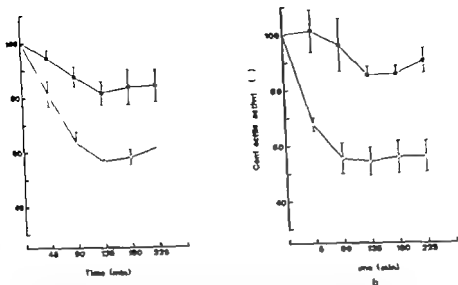
Blockade of the β -adrenoceptors in the myometrium with propranolol (2×10^{-6} M) affected the potassium contracture in the same way as reserpization. Thus, the drug reduced the transient relaxation and caused a better maintained active tension (Fig. 1 c). There was no observable change in the spontaneous contractions in the presence of propranolol.

Prostaglandin E_1 (PGE_1) increased the amplitude of the spontaneous contractions but did not remove the transient relaxation in the depolarized myometrium (Fig. 1 d). (Similar results were obtained with PGE_2).

B Oophorectomized rats treated with estrogen Uterine strips from oophorectomized rats treated with polyestradiol phosphate exhibited no phasic contraction in response to potassium solution, but the tension continued to increase during the first 2–3 min. The tension thereafter remained unchanged or declined slowly. Reserpization of the animals



Fig. 2 Contractile responses of estrogenized rat uterus induced by immersion in a medium containing 10 mM potassium. (a) control, (b) after reserpization, (c) in the presence of 10^{-6} M propranolol, (d) in the presence of 10^{-6} M atropine.



7 a. Effect of repeated activation (127 mM potassium) on the contractile activity of the rat portal vein: control (solid circles) and estrogenized (open circles) animals. Each point represents the mean value and S.E. of 4 experiments.

7 b. Effect of repeated activation (127 mM potassium) on the contractile activity of the rat portal vein: control (solid circles) and estrogenized (open circles) animals. Phenolamine (10^{-6} M) added to the bathing tissue at each series. Each point represents the mean value and the S.E. of 4 experiments.

3 *Oophorectomized rats treated with estrogen.* Fig. 6 shows the average response to tension in estrogenized portal veins. There was a statistically significant reduction of muscle tension during the first 2 min of the contracture of the estrogenized preparation compared with the control. Reserpinization caused a more pronounced relaxation in reserpinized portal veins than in the non-estrogenized tissues (cf Fig. 5). The average response of vessels treated with reserpine coincided with that obtained in the presence of α -methylhistamine (10^{-4} M).

C. *Effect of repeated activation by potassium.* When rat portal veins were subjected to periodic depolarizations with the isotonic potassium solution, the contractile responses declined to a steady level which was reached after about 4 contractions, 135 min (Fig. 7 a). Its decline in contractile response was 20% in the non-estrogenized preparations and 30% in preparations from estrogen treated animals.

The reduction of contractile response in rat portal veins during repeated depolarizations could have been due to a gradual loss of noradrenaline from the intramural nerves. In order to test this, repeated depolarizations were performed in the presence of 10^{-6} M phenolamine (Fig. 7 b). The percentage reduction of the contractile response was about the same as in the absence of α -adrenoceptor blockade (cf Fig. 7 a), although the absolute values were smaller. Again, the responses declined more in preparations from estrogen-treated animals.

D. *Effect of noradrenaline in the depolarized portal vein.* Fig. 8 shows the contractile effect of noradrenaline added to a calcium induced contracture of the depolarized rat portal vein.

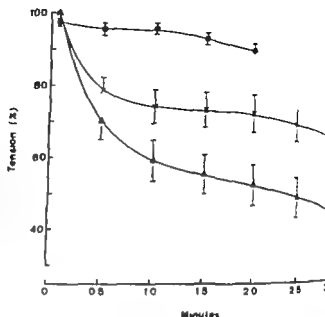


Fig. 5 Average time course of contractions ($n=9$) evoked by 127 mM potassium in isolated portal veins of non-estrogenized rats. Solid circles control. Crosses preparations from reserpinized animals. Triangles preparations from untreated animals with 10^{-8} M phentolamine added to the bathing solution.

Fig. 5 shows the influence of reserpinization and α -adrenoceptor blockade on the course of potassium contractions in the isolated portal vein. After reserpine treatment, there was a more rapid relaxation. 1 min after initiation of the response, the contractile force of the reserpinized preparations had decreased by about 25%. Similar but more pronounced effects were produced by phentolamine (10^{-8} M). The muscular tension decreased by 40% during the first minute of potassium contractions evoked in the presence of phentolamine.

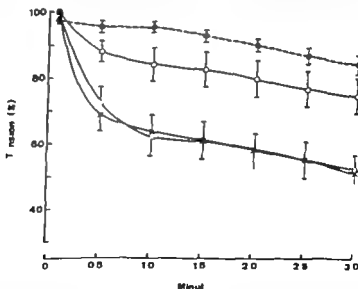


Fig. 6. Average time course of contractions ($n=8$) evoked by 127 mM potassium in isolated portal veins of the rat. Solid circles (broken line) control (no estrogen treatment), same data as in Fig. 5. Open circles estrogenized preparations. Crosses: preparations from estrogen-treated and reserpinized animals. Triangles preparations from estrogenized animals with 10^{-8} M phentolamine added to the bathing solution. Asterisks indicate that the difference between values of control and estrogenized preparations is statistically significant ($p < 0.05$).

er the exper. suggest that reduced sensitivity of the myometrium to noradrenaline not account quantitatively for the finding that estrogen treatment removes the typical influence on the potassium induced contracture. A dose of noradrenaline that maximum (80%) relaxation in the non-estrogenized uterus can be shown to give at 0 relaxation in the estrogenized muscle (Fig. 3). The complete absence of relaxation in the estrogenized uterus therefore suggests that an insufficient amount of noradrenaline ceased to act on the myometrium in the latter case.

er studies of the effects of estrogen on the noradrenaline contents of the rat uterus led to different conclusions. Histochemical data suggest that estrogen treatment reduces the fluorescence of adrenergic nerves in rat myometrium (Hervonen *et al.* 1973, Nová *et al.* 1974), whereas biochemical studies indicate that the total amount of norepinephrine in the rat uterus is independent of estrogen (Oskarnson 1960, Falck *et al.* 1974). e uterine weight is increased by estrogen, the latter results suggest that the quantity of norepinephrine per g of uterine tissue is reduced by estrogen treatment. However even a decrease in the relative quantity of noradrenaline in the estrogenized rat uterus did not explain the complete absence of relaxant effect of intramural noradrenaline after oestrogen treatment, as shown by the present results. It is possible that estrogen inhibits the release of noradrenaline from adrenergic nerve endings.

e prostaglandins E₁ and E₂ have been shown to inhibit noradrenaline release from sympathetic nerves (Hedqvist 1969, Hedqvist and Brundin 1969, Stjärne 1973). However as shown in the present study none of these prostaglandins removed the adrenergic influence on the non-estrogenized myometrium, therefore it seems unlikely that the adrenergic effect of estrogen is mediated through an increased prostaglandin synthesis.

e rat uterus is supplied with cholinergic nerve fibres (Adham and Schenk 1969) but its functions are unknown (Spannau 1975). Atropine had no effect on the potassium induced contracture in the estrogenized myometrium, as shown in the present study. This probably excludes the possibility that estrogen treatment obscures the adrenergic influence by increasing the cholinergic tone of the preparation.

he estrogenized rat portal vein responded to potassium in a similar way as preparations in which the adrenergic influence had been reduced by reserpinization or α -adrenoceptor blockade. Thus it seems likely that estrogen in this organ, as in the rat uterus, has an inhibitory action (cf. Lloyd and Pickford 1961). This action might explain why estrogen treatment reduces the tone of human peripheral veins (Goodrich and Wood 1966).

we judge from the present results, the sensitivity of the α -adrenoceptors of the rat portal vein is not appreciably changed by estrogenization. This suggests that estrogen inhibits adrenergic influence in the rat portal vein by reducing the amount of noradrenaline that acts on the receptors.

It is a pleasure to thank Professor K. A. F. Edman for advice and helpful criticism during the course of this investigation, Professor K. E. Andersson for helpful discussions and criticism of the manuscript and Miss Christina Olsson for skilful technical assistance.

This work was supported by grants from the Swedish Medical Research Council (project No. 04X-184), and from the Medical Faculty, University of Lund.

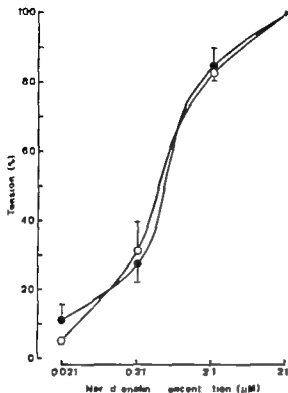


Fig. 3 The contractile effect of noradrenaline on rat portal veins immersed in isotonic potassium solution containing 3 mM calcium. Solid line, control. Open circles, estrogenized tissue. Each point represents the mean and the S.E. of 5 expts.

Maximum contractile effect was attained at a noradrenaline concentration of about 10^{-6} M. Estrogen treatment did not change the sensitivity of the rat portal vein to added noradrenaline.

Discussion

The present study showed that the mechanical response of the rat uterus and portal vein to a potassium-rich medium might be modified by simultaneously released noradrenaline. The evidence in favour of this seems fairly conclusive as blockade of the adrenoceptors resulted in changes almost identical to those induced by noradrenaline depletion with reserpine.

Earlier reports suggested that only the vasculature receives adrenergic innervation in the rat uterus (Norberg and Fredriksson 1966, Sjöberg 1967) but more recent investigations demonstrated that the uterine muscle itself has adrenergic nerves (Adham and Schenk 1971, Hervonen *et al.* 1973, Poskonova *et al.* 1974). The latter observation is consistent with the finding that the contractile activity of the rat uterus *in situ* depends on the adrenergic innervation (Dels and Pickford 1964, Hollingsworth 1974). The marked influence of released noradrenaline on the non-estrogenized rat uterus—shown in the present study—suggests that the myometrium contains noradrenaline stores but does not give any information about the precise location of these stores.

The adrenergic influence on the myometrium of the oophorectomized rat disappears after estrogen treatment (*cf.* Dels and Pickford 1964) as judged from this study. The relative effect of noradrenaline was found to be less pronounced in the estrogenized uterus.

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Judging from the present results, the sensitivity of the α -adrenoceptors of the rat portal vein is not appreciably changed by estrogenization. This suggests that estrogen inhibits the adrenergic influence in the rat portal vein by reducing the amount of noradrenaline released to act on the receptors.

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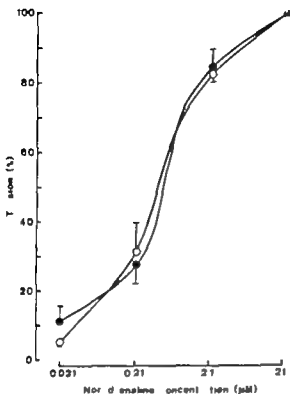


Fig. 8 The contractile effect of noradrenaline on rat portal veins immersed in isotonic potassium solution containing 3 mM calcium. Solid circles: control. Open circles: estrogenized tissue. Each point represents the mean and the SE of six experiments.

Maximum contractile effect was attained at a noradrenaline concentration of about 10^{-4} M. Estrogen treatment did not change the sensitivity of the rat portal vein to added noradrenaline.

Discussion

The present study showed that the mechanical response of the rat uterus and portal vein to a potassium rich medium might be modified by simultaneously released noradrenaline. The evidence in favour of this seems fairly conclusive as blockade of the adrenoceptors resulted in changes almost identical to those induced by noradrenaline depletion with reserpine.

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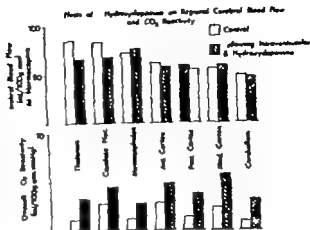
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1 The upper graph illustrates arterial blood flow as a function of arterial CO₂ and blood flow in response of saline (open bars) or 6-hydroxydopamine (filled bars) administered intraventricularly. The lower graph shows the regional cerebral blood flow response to increases in arterial CO₂ in the two experimental groups mentioned.



ality by testing the cerebral CO₂ responsiveness following the intraventricular injection hydroxytryptamine, an agent which is selectively neurotoxic for the catecholamine-releasing neurones (Malkov and Sachs 1968).

Experiments were carried out on 23 adult male Sprague-Dawley rats (300 g, b. wt.), fed and watered *ad libitum*. The rats were divided into 4 groups. The first two groups received an intracerebral injection of saline (20 µl) one of these groups was studied at normocapnia (35–40 mmHg, 5 rats), the other at normocapnia (30–35 mmHg, 7 animals). The last two groups of rats received an injection of 230 µg 6-hydroxydopamine (6-OHDA-HCl in 20 µl saline) into the lateral ventricle and were again studied at either normocapnia (4 rats) or hypertcapnia (6 rats). The intraventricular injections were given stereotactically under light ether anaesthesia (Brevital, Lilly 40 mg/kg) 7 days prior to the blood flow measurements. Various parts of the brains were processed for fluorescence histochemistry according to the Falck-Hillarp formaldehyde method (for technical details see Björklund, Falck and Ögren 1972).

Arterial blood flow was measured with 2–3 catheters as O₂. Following cannulation of the trachea, the animals were intubated artificially and anaesthesia was maintained with 70% N₂O–30% O₂ gas mixture. Body temperature was kept constant at 37°C. Cerebral blood flow was measured by the ¹⁴C-ethanol technique, adapted by Elvén *et al.* (1974). The femoral arteries and veins were cannulated on both sides. One of the femoral catheters was used for the recording of mean arterial pressure, the other was used for the measurement of arterial pCO₂, pO₂, pH and radioactive ethanol concentrations. One femoral catheter was used to inject the tracer (¹⁴C-ethanol, 20 mCi in saline) whilst the other catheter was used to inject saturated KCl for the termination of the blood flow estimation.

The radioactive tracer was infused over 30 s, arterial samples (30 µl) being withdrawn every third second. The animal was sacrificed as soon as the last sample had been obtained. The rat was decapitated and the brain was immediately frozen in liquid nitrogen. At a later stage, seven brain regions (see Fig. 1) were sectioned out, each piece of tissue being dissolved with 0.5 ml Soluene 1–1 (Packard) and 1 ml benzene. The radioactivity in tissue and arterial samples was measured in a standard liquid scintillation counter with quench correction as performed according to established principles.

In each brain region, cerebral blood flow was plotted against arterial CO₂ and a standard linear regression was calculated using the least squares method. The slopes of these regressions yielded the cerebrovascular CO₂ reactivity (in ml/100 g min/mmHg) for each brain region in saline-treated and in 6-OHDA-treated rats. Statistical difference between overall responses was studied by an analysis of variance.

Effects of Intraventricular 6-Hydroxydopamine on Cerebrovascular CO₂ Reactivity in Anesthetized Rats

By

LARS EDVINSSON, JAN ERIK HARDEBO and ERIC T. MACKENZIE

Received 21 April 1977

Abstract

EDVINSSON L., J. E. HARDEBO and E. T. MACKENZIE. *Effects of intraventricular 6-hydroxydopamine on cerebrovascular CO₂ reactivity in anesthetized rats* Acta physiol scand 1977 101 122-125

Regional cerebral blood flow was measured by the C-ethanol technique in anesthetized rats before and after intraventricular injection of 6-hydroxydopamine. This treatment reduced the fluorescence of central noradrenaline and dopamine nerve terminals, as well as of the perivascular nerve terminals of cerebral vessels. The administration of 6-hydroxydopamine had no significant effect on cerebral blood flow at normocapnia. The cerebrovascular reactivity to hypercapnia was significantly increased in the 6-hydroxydopamine treated animals. The results indicate an involvement of central catecholaminergic pathways in the cerebrovascular reactivity to hypercapnia.

The most widely recognized and documented responses of the cerebral circulation is increase in cerebral blood flow secondary to hypercapnia, and the decrease in cerebral blood flow secondary to hypocapnia. Although the exact mechanism of action of arterial CO₂ is unknown, it is believed generally that this action involves pH changes in the interstitial fluid that surrounds cerebral resistance vessels (for review see Lassen 1974). However, one of the experiments would speak against this hypothesis: a number of studies have shown that pontine transections, or ablations can severely attenuate or even completely abolish the cerebral vasodilatory response to induced hypercapnia (Capon 1975, Fenske *et al.* 1967, Shalit *et al.* 1967). The intracarotid infusion of the β -adrenolytic agent, propranolol, similarly reduces the cerebral circulatory reactivity to induced hypercapnia (Mackenzie, McCulloch and Harper 1976).

These observations might suggest that the central catecholaminergic systems—arise from the brain stem—are in some way involved in the cerebrovascular reactivity to changes in arterial CO₂ tension. The purpose of the present investigation was to examine

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Fluorescence histochemistry In those animals treated with 6-OHDA, demonstrated marked overall reduction of the central noradrenaline (NA) and dopamine (DA) terminals. Very few NA and DA nerve terminals remain within the zone 2 mm to ventricular surface. Terminals outside this zone were also clearly reduced. Whole brain NA and DA have been shown to be reduced to 34 and 46%, respectively of control 2 days after a single intraventricular injection of 250 µg 6-OHDA (Iversen and Uretsky 1971). A depletion of the perivascular nerve terminals in the cerebral vessels was noted in the investigation.

The intraventricular administration of 6-OHDA had no significant effect on blood flow at normocapnia ($P > 0.05$ in every brain region, Student's *t*-test for unpaired data). However, the CO_2 reactivity—the slope of the relationship between cerebral flow and arterial CO_2 tension—was significantly increased ($P < 0.01$, analysis of variance between overall regressions).

Although the findings of this investigation indicate that the neurotoxic effects of 6-OHDA to the central catecholaminergic pathways may affect cerebrovascular CO_2 reactivity, interpretation of the physiological significance of these findings is difficult. Firstly it must be recognized that intraventricular 6-OHDA destroys dopaminergic as well as noradrenergic neurones (Iversen and Uretsky 1971, Ungerstedt 1971a). Secondly, the intraventricular administration of 6-OHDA is neurotoxic to the perivascular (sympathetic) nerves, as well as to the central catecholaminergic neurones. This was shown in the present investigation. Nonetheless, it is unlikely that the perivascular sympathectomy contributed materially to the observed response as a number of investigations have shown that neither cervical sympathectomy nor α -adrenergic blockade will influence the cerebrovascular response to hypercapnia (for review see Edvinsson and MacKenzie 1976). The third problem with the interpretation of this preliminary report concerns the development of denervation hypersensitivity. As is the case with peripheral sympathetic nerves, the degeneration of central noradrenergic neurones leads to a denervation hypersensitivity of the pre- and postsynaptic receptors. This is initiated approximately two days after the denervation; the loss of presynaptic terminals is associated with a diminished reuptake of noradrenaline (Trendelenburg 1971). The injection of 6-OHDA into the lateral ventricle causes a specific degeneration of central catecholaminergic nerve terminals and a depletion of catecholamines (Iversen and Uretsky 1971, Ungerstedt 1971a). A central denervation hypersensitivity has been associated with the administration of 6-OHDA on a number of occasions (Huang and Daly 1974, Ungerstedt 1971b). It has been demonstrated recently that the intraventricular administration of 6-OHDA effects an increase in the density of β -receptors in the rat cerebral cortex, without affecting the affinity of the receptors for the β -adrenergic agonist, isoprenaline (Lundberg *et al.* 1976).

Future investigations into the relationship between the central catecholamine pathways and the cerebral circulatory responsiveness to hypercapnia will need to examine the effects of lesions of the noradrenergic or dopaminergic tracts at various intervals, following the administration of the lesions, before this relationship is well understood.

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Effects of Ethanol on Urinary Arginine Vasopressin Excretion in Two Rat Strains Selected for Their Different Ethanol Preferences

By

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Abstract

LINKOLA, J., F. FYHRQUIST and O. FORSANDER. *Effects of ethanol on urinary arginine vasopressin excretion in two rat strains selected for their different ethanol preferences.* Acta physiol. scand. 1977 101 126-128

The effects of ethanol on urinary excretion of arginine vasopressin (AVP), sodium, and potassium were investigated in two rat strains specially selected for their different alcohol preferences. The alcohol preferring (AA) strain excreted more AVP and the water preferring (ANA) strain more urine and sodium during 6 hours after ethanol intubation (2.4 g/kg b.w. 20% v/v). The data is insufficient to establish a causal relationship between differences in water and electrolyte metabolism and voluntary ethanol consumption.

Ethanol affects the water and electrolyte balance in man and laboratory animals (Waltz and Barry 1970). Recently differences have been found in the diuretic behaviour and electrolyte excretion of two rat strains selected for their voluntary ethanol ingestion (Linkola 1976). At the same time, attempts to increase voluntary ethanol consumption of animals inducing changes in this balance have been unsuccessful. Thus, it seemed important to investigate more closely the hormonal regulation of water metabolism in the ethanol preferring AA (Alko Alcohol) strain and water-preferring ANA (Alko, Non-Alcohol) strain (Eriksson 1968, Eriksson 1971).

Urinary excretion of arginine vasopressin (AVP) reflects the plasma concentration of this polypeptide hormone (Miller and Moses 1971). We therefore studied the urinary excretion of immunoreactive AVP in AA and ANA rats after the administration of a single dose of ethanol. Urinary excretion of sodium and potassium was also recorded.

Material and Methods

10 males and 10 females aged 5 months, were taken from each of AA and ANA strains (F₁ generation) for use in the experiments. They had been given standard ASTRA EWOS (Södertälje, Sweden) rat food and water freely until the experiment began. At 9 a.m. they were intubated with 2.4 g/kg ethanol as 20% v/v solution in distilled water. Urine was collected for 6 h as described earlier (Linkola 1976). The collecting tubes were kept in an ice bath.

1. Urine output (UO) and osmolality (Osm), urine arginine vasopressin concentration (AVPc), urinary arginine vasopressin output (AVPo), sodium (Na) and potassium (K) output \pm S.D. during 6 h after ethanol administration (2.4 g/kg b.w. \pm 20% /h) in AA and ANA rats. In both groups 20, if not mentioned, p represents the significance of differences.

UO ml/kg b.w.	Osm mOsm/kg	AVPc pg/ml	AVPo pg	Na mmol/kg b.w.	K mmol/kg b.w.
40.39	1551 \pm 742	143.1 \pm 142.1	265.6 \pm 32.3	0.4.03	1.1.04
$p < 0.001$	$p < 0.001$	$p < 0.005$	$p < 0.05$	$p < 0.001$	$p < 0.10$
115.56	601 \pm 200	36.6 \pm 22.5	137.5 \pm 6.9	1.0.05	1.2.04

Urine vasopressin concentration as measured by radioimmunoassay (F)urquest *et al.* 1976) modified assay of AVP in rat urine. The urine as diluted tenfold with distilled water to avoid non-specific toxic blank tubes (about antiserum yielded values close to zero. Recovery of added synthetic vasopressin (Gibco V1, 100 IU/ml, St. Louis, Missouri) in the range 2.5–45.0 pg/ml as found 85% with no interference as negligible has urine as diluted 1:10 or more. All samples from both rats were assayed at the same time. Urine sodium and potassium concentrations are determined by flame photometer (Eel), and the osmolality by vapor-pressure osmometer (4% error).

Results

Urine output of AVP was approximately 1.9 times higher in the AA strain (Table 1). Difference in urine AVP concentrations was even more significant ($p < 0.005$). Urine output was about 2.3 times greater in the ANA strain than in the AA strain, and urine output about 2.6 times higher in the AA strain than in the ANA strain (Table 1). Urinary output was about 2.5 times higher in ANA than AA rats, but no significant difference observed in the potassium excretion.

Discussion

The hypothesis that disturbances in the water and electrolyte balance may stimulate voluntary alcohol drinking has been tested in animal experiments. The results of Jida (1957) with mice do support the hypothesis, and Balthazart and Montastruc (1962) even observed decreased voluntary ethanol consumption in dogs treated with antidiuretic hormone. Eriksson (1971) showed that two diuretic drugs had no effect on alcohol consumption in rats. A recent report (Lindholm 1976) revealed differences in the water and electrolyte metabolism in rat strains selected by outbreeding to drink, in a free-choice situation, as much ethanol as possible (AA strain) or to avoid ethanol (ANA strain) (Eriksson 1971). Our findings here suggest that the different diuretic behavior of AA and ANA rats can be explained by differences in the antidiuretic hormone release. Antidiuretic hormone release is a consequence of complex events. Among these are the activity of osmoreceptors and the hypothalamic neurones governing the release of vasopressin. Functional differences in these structures may be responsible for strain differences in vasopressin release.

The reason for higher sodium output in ANA rats than AA rats remains unclear. It may be a direct effect of AVP (Finn 1973) or may indicate differences in other controlling systems, located either at the central level (Dorn *et al.* 1970) or in the adrenal cortex (Knochel and White 1973).

It has been observed in rats (Marquis *et al.* 1975) that ethanol causes only a transient suppression of vasopressin release, followed by a rapid increase in AVP production. We suggest that the stimulation of vasopressin release after ethanol administration should be emphasized when the effects of ethanol on water balance are being considered. Further work is needed to investigate the relationships between osmoregulation and voluntary ethanol drinking.

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Neuronal and Extraneuronal Outflow of H Noradrenaline Induced by Electrical-Field Stimulation of an Isolated Blood Vessel

By

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Abstract

SCHJØLD J and O A. NEDERGAARD *Neuronal and extraneuronal outflow of H-noradrenaline induced by electrical-field stimulation of an isolated blood vessel.* Acta physiol. scand. 1977 101 129-143

The adrenergic neuronal selectivity of ^3H -outflow elicited by electrical-field stimulation of rabbit isolated coronary artery preloaded with H-(+)-noradrenaline (^3H -NA) was examined. Following incubation (5 min) of arterial rings at 37°C with ^3H -NA (10^{-6} M) and 100 min wash-out period, the sympathetic nerves were stimulated selectively and experimentally as regards tension development, with constant current (250 μA , 300 monophasic pulses, 10 Hz, 0.3 ms). The neural stimulation-induced ^3H -outflow is greater than the subsequent 5 outflows, each remained almost constant (79, 20, 19, 17, 15 and 16 pmol g^{-1} respectively). ^3H -NA and H-O methylated plus deacetylated metabolites constituted 11% and 74%, respectively of the total ^3H -outflow induced during the neural stimulation period, but 35% and 38%, respectively during the second stimulation period. Omission of Ca^{2+} in the physiological salt solution altered the 6 stimulation-induced outflows to 67, 40, 35, 24, 21 and 21%, respectively of the untreated repetitions. Bretylum ($3 \cdot 10^{-4}$ M) or tetrodotoxin (10^{-6} M) reduced the ^3H -outflow to approximately 50% of the untreated. Stimulation-induced outflow from artery rings preloaded with ^3H -NA in the presence of cocaine (10^{-4} M) or in the cold (15°C) approximated the Ca^{2+} , bretylum and tetrodotoxin-insensitive release. These treatments all completely blocked the neurogenic contractile response. When artery rings were preloaded with ^3H -NA in the presence of norepinephrine (10^{-4} M), the stimulation-induced Ca^{2+} -sensitive outflows were markedly reduced. The results suggest that the field stimulation-induced release of ^3H -NA is in part of extraneuronal origin.

Key words: Bretylum, calcium deprivation, cocaine, cold, electrical-field stimulation, extraneuronal release, neuronal release, ^3H -noradrenaline release, pulmonary artery tetrodotoxin.

The *in vivo* release of neurotransmitter from postganglionic sympathetic neurones elicited by nerve stimulation is commonly studied by labelling the preparation and presumably the adrenergic neurones with radioactive noradrenaline (^3H -NA). This method involves the following steps. (1) loading of the tissue with ^3H -NA (2) a wash-out period, in which supposedly non-specifically bound ^3H -NA is removed, (3) stimulation of the adrenergic neurones by electrical-field stimulation (4) fractional collection of ^3H -outflow and (5)

determination of total tritium or ^3H NA and its H -metabolites by column chromatography and liquid scintillation counting. This elaborate method has been used for several blood vessels: the rabbit main pulmonary artery (Su and Bevan 1970, Nedergaard and Schrold 1973, Starke *et al* 1974, 1975), rabbit aorta (Bevan *et al* 1972, Schrold and Nedergaard 1976), rabbit ear artery (Allen *et al* 1973), dog arteries and veins (Vanbrunt *et al* 1973, Vanhoutte 1974), human blood vessels (Stjärne and Gripe 1973, Stjärne and Brundin 1975).

The utility of this method rests on the assumption *inter alia* that the radioactive NA binds selectively at the site of transmitter storage and released from it upon nerve stimulation (Oswald 1976). The selectivity of the neuronal origin can be ascertained by means, e.g. tetrodotoxin (TTX) an agent which selectively blocks axonal conduction in some smooth muscle preparations (Gershon 1967) or bretylium, an adrenergic neurone blocking agent. It has been reported that electrical-field stimulation induced H -outflow was invariably abolished in the rabbit pulmonary artery by TTX and bretylium (Su and Bevan 1970). Using the same artery we (Nedergaard and Schrold 1973) and others (Starke *et al* 1974, 1975) used stimulation that caused supramaximal excitation of adrenergic neurons with respect to tension development. However neither of these authors specified where the H -outflow represented selective transmitter release from adrenergic neurones. To our surprise we have later found that bretylium did not fully abolish the stimulation-induced H -outflow.

There is another puzzling aspect of the stimulation induced H -outflow from rabbit pulmonary artery preloaded with ^3H NA: a marked decrease in the amount of released radioactivity occurs with successive stimulations of the preparation, although the contractile tension responses remain stable (Su and Bevan 1970, Nedergaard and Schrold 1973).

We therefore decided to examine in detail the neuronal selectivity of the H -outflow elicited by electrical-field stimulation of the rabbit pulmonary artery preloaded with ^3H -NA. Our results indicate that the H -outflow represents release from neuronal and extraneuronal sites.

Preliminary reports of this work were presented at the Sixth International Congress of Pharmacology, Helsinki (Schrold and Nedergaard 1975 a) and at the Second International Symposium on Vascular Neuroeffector Mechanisms, Odense (Schrold and Nedergaard 1975 b).

Methods

Drugs. The following drugs were used: Bretylium tosylate (B. roughs Wellcome and Co., Wellesbourne, U.S.A.), cocaine hydrochloride, 3,4-dihydroxyphenylglycol (DOPEG, Sigma, Saint Louis, U.S.A.), (-)-noradrenaline hydrochloride (NA, Fluka, Buchs, Switzerland), (-)-7 H noradrenaline (^3H -NA, 9.2 Ci/mmol, The Radiochemical Centre, Amersham), (\pm)-normetanephrine hydrochloride (NMN, Sigma, Saint Louis, U.S.A.) and tetrodotoxin (TTX, Sigma, Saint Louis, U.S.A.). The purity of ^3H NA was checked by alumina column chromatography and was found to be above 90% pure.

Self solution. The composition (mM) of the physiological salt solution (PSS) was: Na^+ 144, K^+ 4.9, Ca^{2+} 1.3, Mg^{2+} 1.2, Cl^- 126.7, HCO_3^- 25.0, SO_4^{2-} 1.2, glucose, 11.1, ascorbic acid 0.11 and calcium disodium ethylenediaminetetraacetic acid (CaNa_2EDTA), 0.03. Ca^{2+} -free PSS: Calcium (chloride) is omitted and CaNa_2EDTA was replaced by Na_2EDTA (0.03 mM). The solution was aerated with oxygen and had pH 7.4.

Preparation. A modification of the method described previously was used (Nedergaard and Schrödl 1973). A small pulmonary artery was excised from anaesthetized cross-clamped rabbits. It was cleaned of excess adipose and perivascular tissue and lacerated in PEK. The artery was divided into 2 almost equal sized rings. It was dissected in the following proximal and distal with reference to these positions relative to the artery. Each ring was suitably mounted on plastic holder and connected by platinum suture to electrode. Each tissue was placed in a jacketed plastic bath filled with PEK (2.0 ml) maintained at 37°C . Each bath could independently be emptied and filled with prewarmed (37°C) PSS at a constant time rate.

Stimulation. The rings were subjected to electrical-field stimulation using Grass S48 square wave stimulator in connection with constant current unit (S. Jacobsen, Odense University). The unit was gated by the Grass stimulator and ensured that the current flow (250 mA) delivered as constant gradient of the variable impedance existing in the tissue bath. In preliminary experiments, it was observed that the impedance varied from bath to bath and for the same bath from preparation to preparation. This variability is in part probably related to the geometric arrangement of the tissue relative to the stimulating electrodes. Two platinum electrodes were placed on either side of the arterial ring. Each preparation was stimulated four to six times (Q_1 to Q_6) in standard manner (trials of 300 monophasic pulses in duration, 0.3 ms , frequency 10 Hz ; current, 250 mA) with rest period of 13.5 min between each stimulation period. Pulses were monitored on an oscilloscope (Hewlett Packard 1201 A). The stimulation electrodes provided experimental stimulation of adrenergic nervous fibres as regards the contractile power (Nedergaard and Schrödl 1973).

Recording. The isometric contractions of the vascular smooth muscle were recorded by means of a strain gauge displacement transducer (Statham, G10B $\pm 0.15-300$) connected to a linear pen recorder (Sargentich, model 870). The rings were stretched at 3 g tension for 10 min and subsequently maintained at 1 g ring tension. The preparations were allowed to rest for at least 20 min before proceeding. At the end of each experiment, NA ($2.5 \cdot 10^{-6} \text{ M}$) was added to the PEK in order to elicit the maximal obtainable contraction response.

Fractionation. The arterial rings were incubated with ^3H NA (10^{-6} M) for 45 min. Then the bath was suitably emptied and refilled with PEK (1.75 ml) every 2 min for the remainder of the experiment. The rings are stimulated for the first time (Q_1) after wash-out period of 100 min (vide supra). The bath fluid is collected directly in counting vials by means of a fraction collector (J. Cappelet, Odense University). At the end of the collection period the rings were removed from the bath and cut open into rectangles. These are blotted under pressure (50 g) for 10 sec on filter paper previously moistened with PEK, transferred to 25 ml polyethylene liquid scintillation counting vial and the wet weight (range $12.9-19.5 \text{ mg}$) is determined. After addition of 0.50 ml Protosol (Nuclear Chicago Corporation) the closed vial is allowed to stand at room temperature to allow for partial desiccation of the tissues. Then each tissue-solubilizing solution was added 18 ml of fluor solution with the following composition: Liquasol (Packard), 42 ml, benzene methanol, reagent grade, 300 ml and toluene, reagent grade to 1 liter. Then the fraction-containing vials are added 16 ml of scintillation fluid composed as follows: 2,5-diphenyloxazole (PPO, Hopkin & Williams, Chadwell Heath, England), 3.0 g; 1,4-di-(2-(4-methyl-5-phenyl-1,3,4-oxadiazolyl))-benzene (Dimethyl-POPOP, Krich Light Laboratories, Colbrook, England), 0.1 g, Triton X 100, technical grade, 333 ml and toluene, technical grade, to 1 liter.

Tritium was measured with liquid scintillation spectrometer (Mark II, Nuclear Chicago Corporation). Counting was determined by adding known amounts of $^3\text{H}_2\text{O}$ or ^3H -toluene to representative samples. Scintillation-coated peak ^3H -outflow was calculated by summation of each fraction value which entered in the formation of the peak across estimated positive outflow. The latter was calculated by using the ratio value of the raw fractions obtained just prior to each stimulation. The outflow of tritium was corrected for tissue weight, counting efficiency and specific activity and expressed as pmol g^{-1} tissue or as per cent of total tritium content in the tissue at the onset of each stimulation. The latter was calculated by summation of measured tritium outflow and tritium content at the end of an experiment.

Determination of ^3H -NA and ^3H -metabolites

In the experiments involving determination of ^3H -NA and its ^3H -metabolites only 2 periods of stimulation (Q_1 and Q_2) are applied. The content of ^3H -NA, ^3H -DOPEG, ^3H -OMDA and total tritium was measured in the two fractions just before each stimulation (pre-stim: P' and P'') and in the fraction during stimulation (Q).

determination of total tritium or ^3H NA and its ^3H -metabolites by column chromatography and liquid scintillation counting. This elaborate method has been used for several blood vessels: the rabbit main pulmonary artery (Su and Bevan 1970, Nedergaard and Schrold 1973, Starke *et al.* 1974, 1975), rabbit aorta (Bevan *et al.* 1972, Schrold and Nedergaard 1976), rabbit ear artery (Allen *et al.* 1973), dog arteries and veins (Vanhoutte *et al.* 1973, Vanhoutte 1974), human blood vessels (Stjärne and Gripe 1973, Stjärne and Brundin 1975).

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Salt solution. The composition (mM) of the physiological salt solution (PSS) was: Na^+ 144.2, K^+ 4.9, Ca^{++} 1.3, Mg^{++} 1.2, Cl^- 126.7, HCO_3^- 25.0, 50mM glucose, 11.1 ascorbic acid, 0.11 and calcium disodium ethylenediaminetetraacetic acid (CaNa_2EDTA), 0.03. Ca^{++} free PSS CaCl_2 (chloride) was omitted and CaNa_2EDTA was replaced by Na_2EDTA (0.03 mM). The solutions were aerated with 5% carbon dioxide (v/v) in oxygen and had pH 7.4.

Preparation. A modification of the method described previously as used (Niedergaard and Schrödl 1977) to expose pulmonary artery was excised from unconscious extraneurated rabbits. It was cleaned of excess fat and perivascular tissue and immersed in PSS. The artery was divided into 2 almost equal sized rings, each one denoted as the following proximal and distal with reference to their position relative to the preparation. Each ring was suitably mounted on plastic holder and connected by platinum stirrups to a perfusion system. Each tissue was placed in a jacketed plastic bath filled with PSS (2.0 ml) maintained at 37°C. The bath could automatically be emptied and filled with prewarmed (37°C) PSS at constant time interval.

Stimulation. The rings were subjected to electrical-field stimulation using Grass S48 square wave stimulator in connection with constant current sink (S. Jacobsen, Odense University). This unit as regulated by the Grass stimulator and ensured that the current flow (150 mA) delivered as constant independent of the variable impedance existing in the tissue bath. In preliminary experiments, it was observed that the impedance varied from bath to bath and for the same bath from preparation to preparation. This variability is in part probably related to the geometric arrangement of the tissue relative to the stimulating electrodes. Two platinum electrodes were placed on either side of the arterial ring. Each preparation was stimulated four to six times (S_1 to S_4) in standard manner (train of 300 monophasic pulses, pulse duration, 0.3 ms, frequency 10 Hz; current, 230 mA) with rest period of 13.5 min between each stimulation period. Pulses were monitored on an oscilloscope (Hewlett Packard 1201 A). The stimulation electrodes provided suprathreshold stimulation of adrenergic neurones (fibres as regards the contractile response (Niedergaard and Schrödl 1977).

Recording. The isometric contractions of the vascular smooth muscle are recorded by means of strain gauge displacement transducer (Ratnam, G10B $\pm 0.15-350$ g) connected to linear pen recorder (Sargent Welch, model SRG). The rings were stretched at 3 g tension for 10 min and subsequently maintained at 1 g resting tension. The preparations were allowed 15 min rest for at least 20 min before proceeding. At the end of each experiment, NA (2.5×10^{-6} M) was added to the PSS in order to elicit the maximal obtainable contraction response.

Effluent. The arterial rings were incubated with H-NA (10^{-6} M) for 45 min. Then the bath was periodically emptied and refilled with PSS (1.75 ml) every 2 min for the remainder of the experiment. The rings were stimulated for the first time (S_1) after wash-out period of 100 min (rule paper). The bath fluid was collected directly in counting vials by means of fraction collector (J. Crypkins, Odense University).

At the end of the collection period the rings were removed from the bath and cut open into rectangles. These were blotted under pressure (50 g) for 10 sec on filter paper previously moistened with PSS, transferred to 25 ml polychlorinated biphenyl scintillation counting vial and the wet weight (range: 12.9-29.5 mg) was determined. After addition of 0.50 ml Protosol (Nuclear Chicago Corporation) the closed vials stood overnight at 30°C to allow for partial dissolution of the tissue. To each tissue-containing vial was added 18 ml of fluor solution with the following composition: Liquifluor (Packard), 42 ml; toluene, analytical grade, 300 ml, and sodium, analytical grade to 1 liter. To the fraction-containing vials were added 16 ml of scintillation fluid composed as follows: 2,5-diphenylazole (PPO; Hopkin & Williams, Chesham Heath, England), 5.0 g; 1,4-di-(4-methyl-5-phenylcarbazolyl)-benzene (Dimethyl-UFOPOP; Koch Light Laboratories, Colnbrook, England), 0.1 g; Triton X 100, technical grade, 333 ml and toluene, technical grade, to 1 liter.

Tritium was measured with liquid scintillation spectrometer (Mark II, Nuclear Chicago Corporation). Quenching was determined by adding known amounts of ³H₂O or H-toluene to representative samples. Scintillation-induced peak ³H-efflux as calculated by summation of each fraction value back entered in the formation of the peak means estimated passive outflow. The latter as calculated by using the mean values of the fractions obtained just prior to each stimulation. The outflow of tritium was corrected for tissue weight, counting efficiency and specific activity and expressed as pmol g⁻¹ tissue or as per cent of initial tritium content in the tissue at the onset of each stimulation. The latter was calculated by summation of initial tritium outflow and tritium content at the end of an experiment.

Determination of H-NA and its H-metabolites

In the experiments involving determination of ³H-NA and its ³H-metabolites only 2 periods of stimulation (S_1 and S_2) were applied. The content of ³H-NA, ³H-DOPEG, ³H-OMDA and total tritium was measured in the two fractions just before each stimulation (passive efflux P' and P'') and in the fractions during stimulation (S_1).

Each of the two-minute fractions (P, P' and S) was collected in cooled (0°C) tubes. Total tritium determined by removing an aliquot (100 µl). To each cooled fraction was added 20 µg each of NA, DOPE and NMN as carriers. The fractions were acidified with acetic acid (1 N) to pH 3-4 and frozen overnight before being submitted to chromatographic analysis.

³H NA and its ³H metabolites were separated by the method of Grafte *et al.* (1973), which was slightly modified. Al₂O₃ was activated according to the method of Well-Malherbe (1971). Each alumina column (diameter 5 mm) was prepared beforehand by washing 200 mg of activated Al₂O₃ with 5 ml sodium acetate (0.1 M), pH 8.2. The fractions were thawed and after addition of 0.1 ml Na₂EDTA (10⁻³ M) they were adjusted to pH 8.2 with NH₄OH (2.5% and 0.25% v/v) during continuous stirring. Immediately thereafter each fraction was poured onto an alumina column, which was washed successively with 1 ml sodium acetate (0.2 M) and 2 ml H₂O. The effluent and subsequent washings from the column contain ³H-methylated (NMN) and ³H O-methylated plus deaminated metabolites (VMA and MOPEG).

Each column was now placed over a Dowex column (50W x 4 15 x 5 mm H⁺, pH, 2.5), which had been washed beforehand with 1 ml H₂O. ³H NA and ³H DOPEG were eluted from the alumina column by separate additions of 1 ml acetic acid (0.5 N). The eluate dripped directly onto the Dowex column.

H DOPEG, a neutral catechol, was then found in the effluent and first washing (2 ml H₂O) from the Dowex column. The latter was washed further with 4 ml H₂O which was discarded. H NA was then eluted from the column by repeated additions (4 times) of 1 ml HCl (2 N). H DOMA was not determined.

The H-content of various collected effluents and eluates (³H-OMDA, ³H NA, ³H DOPEG) was determined by removing 2 ml aliquots and subjecting them to liquid scintillation spectrometry.

The recovery of NA, DOPEG and OMDA, the latter estimated by determination of NMN, is fairly constant. The mean recovery ± S.E. was as follows: NA 80.7 ± 0.7% (n = 22), DOPEG 85.5 ± 1.4% (n = 14) and NMN 97.6 ± 0.9% (n = 14). All data were corrected for these recoveries.

The outflow of H NA and its ³H-metabolites evoked by field stimulation was calculated as a percentage of induced total tritium according to the following formula.

$$\frac{S - \frac{1}{2}(P + P')}{S_T - \frac{1}{2}(P_T + P'_T)} \times 100$$

where P and P' represent radioactivity in the two passive effluent fractions just prior to stimulation, S and S' the H-content in the fraction during stimulation. Subscripts % and T represent radioactivity of ³H NA or ³H-metabolites and total tritium, respectively. All data were converted to dpm.

Statistical analysis. Student's t-test (paired or unpaired samples) or analysis of variance was used to compare differences between means.

Results

Effect of electrical-field stimulation on H-outflow and concomitant contractile response

Controls. The stimulation induced H-outflow expressed either as pmol g⁻¹ (Fig. 1) or as of tissue H-content (Table I), declined markedly from the first to the second period of electrical-field stimulation (P < 0.01). The H-outflows evoked by stimulation periods 2-4 remained approximately constant. In contrast, the concomitant tension response expressed either in gram (Fig. 1) or as % of maximal obtainable response to NA (Table I) increased initially (P < 0.01 S₁ versus S₂, P < 0.05 S₂ versus S₃). Thereafter the tension response remained constant.

There was no difference (P > 0.05) between each of the stimulation-induced H-outflows (S₁ to S₄) from proximal and distal rings, except that the H-outflow as % was larger from proximal rings than that from distal rings during S (Fig. 1 Table I). The tension response in gram obtained with proximal rings was always significantly higher (P < 0.01 S₁ to S₂, S₂ to S₃, S₃ to S₄).

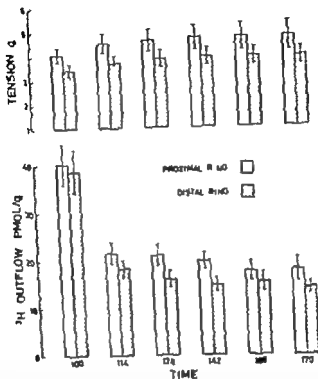


Fig. 1. Effect of electrical-field stimulation on tension response and concomitant H -outflow from rabbit aortic polyanery artery preloaded with ^3H -NA. Ordinate (upper graph): Mean contractile tension (g) (upper graph); Mean stimulation-induced ^3H -outflow (pmol/g) (lower graph). Abscissa: Time (ms) after commencement of wash-out period. Open columns, proximal ring; cross-hatched columns, distal rings. The passive H -outflows (pmol/g $\times 2 \text{ min}^{-1}$) before S_1 and S_2 are (mean \pm S.E.): 16.7 ± 1.9 and 10.8 ± 1.1 (proximal ring) and 16.3 ± 1.6 and 10.3 ± 1.0 (distal ring). The tissue H -contents before S_1 and S_2 are given in Table I. Vertical bars denote S.E. Each column is the mean of 6 determinations.

$P < 0.05$, S_1 to S_4) than that obtained with distal rings (Fig. 1). However the response of proximal and distal rings was found to be equal when the tension development was expressed as % of maximum (Table I).

Ca^{2+} depletion. Omission of Ca^{2+} from the PSS caused a complete inhibition of the contractile response to electrical-field stimulation (S_1 to S_6). However H -outflows were still induced (Fig. 2, Table II). The initial outflow (S_1) was 4 to 8 times higher than the subsequent outflows (S_2 to S_6 , $P < 0.01$, S_1 to S_2 to S_6). The latter decreased slightly with time. In two instances (S_1 and S_2) the H -outflows from distal rings were larger than that from proximal rings (Table II). In the four others there was no difference ($P > 0.05$).

In 4 experiments (data from proximal and distal rings pooled) the rings were stimulated an additional time (S_7) using 1000 pulses instead of 300. Raising the number of pulses increased the H -outflow to $8.5 \pm 1.3 \text{ pmol g}^{-1}$ or $0.43 \pm 0.08\%$ of the tritium content of the artery rings, i.e. significantly higher ($P < 0.05$) than S_6 (see Table II).

When the stimulation-induced H -outflow from control preparations was subtracted the

TABLE I Electrical-field stimulation induced H -outflow and concomitant contractile response of isolated pulmonary arteries. Artery rings were incubated with H NA (10^{-6} M) for 45 s. After a 100 min wash-out period the rings were stimulated 6 times (S_1 to S_6).

Ring		Proximal	Distal		
Tissue ³ H-content					
S ₁		2 100 ± 252 pmol g ⁻¹	1 951 ± 231 pmol g ⁻¹		
S ₆		1 495 ± 194 pmol g ⁻¹	1 390 ± 180 pmol g ⁻¹		
Passive ³ H-outflow ^b					
S ₁		0.80 ± 0.03 %	0.85 ± 0.03 %		
S ₆		0.74 ± 0.04 %	0.77 ± 0.03 %		
Treatment	Time ^c	H-outflow ^d %	Δ Tension %	H-outflow %	Δ Tension
Field stimulation					
S ₁	100	1.93 ± 0.06	37.0 ± 3.3	1.98 ± 0.04	32.7 ± 2.5
S ₂	114	1.20 ± 0.03	42.3 ± 4.2	1.03 ± 0.04	37.3 ± 3.3
S ₃	128	1.18 ± 0.10	44.2 ± 4.9	0.97 ± 0.10	39.7 ± 3.1
S ₄	142	1.31 ± 0.10	43.0 ± 5.4	0.95 ± 0.05	39.8 ± 3.9
S ₅	156	1.13 ± 0.09	44.5 ± 6.0	1.02 ± 0.03	40.5 ± 4.1
S ₆	170	1.20 ± 0.08	44.3 ± 6.3	0.97 ± 0.09	40.3 ± 4.1
NA ^f	184	10.8 ± 0.79	100	10.9 ± 0.64	100

^a Tissue H -content just before (100 min) S_1 and (170 min) S_6 .

^b Passive 3H -outflow expressed as % of tissue H -content just before (96–100 min) S_1 and (166–170 min) S_6 (Time 0 for onset of wash-out).

^c Stimulation-induced H -outflow expressed as % of tissue H -content at the start of the corresponding stimulation period.

^d Stimulation-induced tension response expressed as % of that obtained with a maximally effective concentration ($2.5 \cdot 10^{-6}$ M) of NA.

^e H -outflow elicited by exogenous NA ($2.5 \cdot 10^{-6}$ M).

Values represent mean \pm S.E. ($n=8$).

mean H -outflow value found during the equivalent period of stimulation in Ca^{2+} -free (column 1 minus column 2 in Fig. 2), the stimulation induced 3H -outflow sensitive to Ca^{2+} deprivation was determined to be (mean \pm S.E.) 13.1 ± 3.5 (S_1) 12.2 ± 1.5 (S_2) 12.5 ± 1.3 (S_3) 13.3 ± 1.7 (S_4) 12.7 ± 1.6 (S_5) and 13.0 ± 1.6 (S_6). There was no difference ($P > 0.05$) between these H -outflows (S_1 to S_6).

Effect of bretylium and tetrodotoxin

Bretylium (3×10^{-6} M) and TTX (10^{-6} M) reduced the H -outflow evoked by stimulation (S_1 to S_6) to approximately the same extent as Ca^{2+} -deprivation did (Fig. 2). The tissue H -content was smaller in both the bretylium and TTX-treated preparations compared with that of the rings in Ca^{2+} -free PSS (Table III). Thus, the H -outflow expressed as a % of tissue H -content was higher than that in Ca^{2+} -free PSS. Furthermore, the H -outflow expressed as % of tissue content during S_1 to S_6 was rather constant and decreased less in Ca^{2+} -free medium (Table III).

Bretylium and TTX caused a total inhibition of the contractile response to stimulation while the response to exogenous NA was equal to that obtained with control preparations. This confirmed that the field stimulation did not excite the vascular smooth muscle its

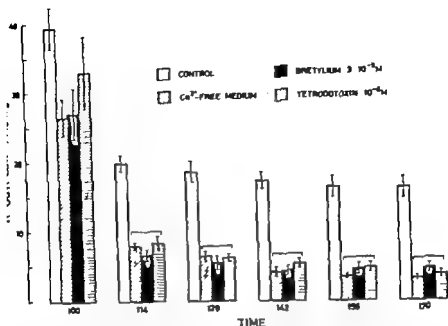


Fig. 2 Effect of calcium deprivation, bretylium or tetrodotoxin on electrical-field stimulation induced H -outflow from rabbit isolated pulmonary artery preloaded with ^3H -NA. Ordinate: Mean stimulation-induced outflow (pmol g^{-1}). Abscissa: Time (min) after commencement of sub-out period. Bretylium (10^{-5} M) and tetrodotoxin (10^{-6} M) are added 100 and 40 min, respectively prior to S_1 (100 min). Ca^{2+} -free medium: The normal PSS was switched to Ca^{2+} -free PSS at the beginning of the passive efflux trial (0 min). All treatments were maintained during the remainder of the expn. The passive H -efflux (mol $\text{g}^{-1} \text{ sec}^{-1}$) before S_1 and S_2 were (mean \pm S.E.): 16.5 ± 1.2 and 10.6 ± 0.7 (control); 25.5 ± 1.5 and 19.0 ± 0.6 (Ca^{2+} -free); 17.9 ± 1.6 and 11.8 ± 1.2 (bretylium); and 22.9 ± 0.8 and 13.3 ± 0.5 (TTX). The mean H -contents before S_1 are given in Table III. Each column is the mean of 3–12 determinations: control, 12 (the results from proximal and distal rings in Fig. 1 were pooled); Ca^{2+} -free PSS, 10; bretylium, 5; tetrodotoxin, 5. Vertical bars denote \pm S.E. The level of significance for the differences between test and control data is indicated: No symbol, Not significant, $P > 0.05$; $P < 0.05$; $P < 0.01$; $P < 0.001$.

Effect of cold, cocaine and cocaine plus normetanephrine

Electrical-field stimulation of artery rings preloaded with ^3H -NA in the cold (3°C) or in the presence of cocaine (10^{-4} M) caused ^3H -outflow (pmol g^{-1}) (Fig. 3), which was of the same magnitude as that found in Ca^{2+} -free PSS (Fig. 2). When NMN (10^{-4} M) was present besides cocaine (10^{-4} M) the outflows were further reduced, but only significantly in the periods related to S_2 and S_3 .

The ^3H -outflows expressed as % of tissue ^3H -content (not shown) were larger following these treatments than those obtained in Ca^{2+} -free PSS. However the tissue ^3H -content is only about 25% of that found with preparations in Ca^{2+} -free PSS (see legend Fig. 3).

Effect of normetanephrine

When NMN (10^{-4} M) was present during the preloading of the arteries with ^3H -NA it markedly reduced the stimulation-induced ^3H -outflows in Ca^{2+} -free PSS. This is apparent

TABLE II Effect of calcium deprivation on electrical-field stimulation induced ^3H -outflow from isolated pulmonary arteries. Artery rings were incubated with ^3H NA (10^{-4} M) for 45 min normal PSS. Then, the PSS was replaced by Ca^{2+} -free PSS containing Na_2EDTA (3×10^{-4} M) which was used throughout the remainder of the expt. The treatment schedule was described in Table I

Ring	Proximal		Distal	
Tissue ^3H -content ^a				
S_1	630 \pm 346 pmol g ⁻¹		2 529 \pm 212 pmol g ⁻¹	
S_6	1 909 \pm 297 pmol g ⁻¹		1 870 \pm 181 pmol g ⁻¹	
	pmol g ⁻¹ 2 min ⁻¹ %		pmol g ⁻¹ 2 min ⁻¹	
Passive ^3H -outflow ^b				
S_1	27.1 \pm 5		23.9 \pm 1.7	0.95 \pm 0.07
S_6	14.6 \pm 1.0		13.3 \pm 0.7	0.75 \pm 0.08
Treatment	Time ^c	^3H -outflow ^d		
		pmol g ⁻¹	%	pmol g ⁻¹
Field stimulation				
S_1	100	21.7 \pm 3.7	0.87 \pm 0.14	31.2 \pm 2.4
S_6	114	7.30 \pm 0.84	0.33 \pm 0.07	8.63 \pm 0.73
S_6	128	5.73 \pm 1.08	0.27 \pm 0.06	7.29 \pm 0.63
S_1	142	2.77 \pm 0.55	0.16 \pm 0.06	5.62 \pm 0.58
S_6	156	3.31 \pm 0.35	0.19 \pm 0.03	3.71 \pm 0.60
S_6	170	2.74 \pm 0.48	0.15 \pm 0.04	3.78 \pm 0.36
NA	184	2.34 \pm 0.30	12.4 \pm 0.58	187 \pm 40
				110 \pm 18

^a Tissue ^3H -content just before (100 min) S_1 and (170 min) S_6 .

^b Passive ^3H -outflow expressed either as pmol g⁻¹ 2 min⁻¹ or as % of tissue ^3H -content just before (96–100 min) S_1 and (166–170 min) S_6 .

^c Time after onset of wash-out.

^d Stimulation-induced ^3H -outflow expressed either as pmol g⁻¹ or as % of tissue ^3H -content at the start of the corresponding stimulation period.

^3H -outflow elicited by exogenous NA (2.5×10^{-4} M).

Values represent mean \pm S.E. (n = 5).

from Fig. 4 as regards the absolute amounts released, but it also applied to the outflow expressed as a percentage of the tissue ^3H -content although the ^3H -content just before^a was lower in experiments with NMN than in those without (Table III)

H NA and its *H*-metabolites

In a separate series of expts. ^3H NA and some of its ^3H -metabolites were determined. ^3H NA, ^3H DOPEG and ^3H -OMDA constituted each approximately 15%, 15% and 40%, respectively of the total tritium in the passive efflux (Table IV). There was no difference between the distribution of the total tritium on ^3H NA and its ^3H -metabolites in the passive efflux prior to S_1 and S_6 , respectively (Table IV). In contrast during electrical field stimulation at 3 Hz for 100 s the distribution of the net ^3H -outflow on ^3H NA and its ^3H -metabolites elicited by S_1 was quite different from that evoked by S_6 . Thus, 11% of the S_1 induced ^3H -outflow was collected as ^3H NA compared to 38% during S_6 , whereas ^3H -OMD

EX II The effect of calcium deprivation, bretylium, tetrodotoxin or normetanephrine on electrical-field stimulation induced ^3H -outflow from rabbit pulmonary arteries. Artery rings were incubated with ^3H -NA (10^{-6} M) for 45 min in either normal PSS or PSS which contained normetanephrine (10^{-6} M). The latter was added 20 min before ^3H -NA. Then the tissues were washed with either normal or Ca^{2+} -free PSS. Bretylium and TTX were added to the PSS during the wash-out period. Treatments were continued for the remainder of the exp. After 100 min wash-out period the tissues were stimulated 4 or 6 times (S_1 to S_6).

treatm	Tissue H content ^a $\mu\text{mol g}^{-1}$	Passive ^3H outflow	^3H -outflow (%) induced by field stimulation ^a					
			S_1	S_2	S_3	S_4	S_5	S_6
exp	2.82 ± 1.63	0.82 ± 0.02	1.93 ± 0.04	1.11 ± 0.04	1.08 ± 0.08	1.08 ± 0.07	1.07 ± 0.05	1.12 ± 0.06
Ca^{2+} -free	NS							
5	10.2579 ± 1.92	1.01 ± 0.05	1.07 ± 0.13	0.36 ± 0.05	0.31 ± 0.04	0.23 ± 0.04	0.20 ± 0.03	0.18 ± 0.02
bretyl	NS	NS	NS					
1	4.1393 ± 2.49	1.13 ± 0.12	1.75 ± 0.15	0.49 ± 0.03	0.46 ± 0.12	0.39 ± 0.09	0.41 ± 0.03	0.38 ± 0.04
tetrodotoxin	NS	NS	NS					
1	3.1706 ± 1.15	1.34 ± 0.13	1.93 ± 0.32	0.36 ± 0.10	0.46 ± 0.07	0.43 ± 0.08	0.42 ± 0.09	0.37 ± 0.06
Ca^{2+} -free PSS + normetanephrine	NS							
1/6	$1.512 \pm .96$	1.31 ± 0.13	0.48 ± 0.06	0.14 ± 0.02	0.09 ± 0.02	0.12 ± 0.02		

Values ^3H -content just before (100 min) S_1 .

Values H -outflow expressed as % of tissue ^3H -content just before (96-100 min) S_1 .

Stimulation-induced H -outflow expressed as % of tissue ^3H -content at the start of the corresponding stimulation period.

The artery rings were stimulated 6 times (S_1 to S_6) at 100, 114, 128, 142, 156 and 170 min after the onset wash-out (cf. Tables I and II).

Control PSS: The results from prewashed and distal rings in Table I are pooled.

Ca^{2+} was omitted from the PSS. The results from prewashed and distal rings in Table II were pooled.

Bretylium (10^{-6} M) was added 100 min before S_1 .

Tetrodotoxin (10^{-6} M) was added 40 min before S_1 .

Ca^{2+} -free PSS and normetanephrine (10^{-6} M).

The data in the horizontal columns were compared with those obtained in Ca^{2+} -free PSS.

bars represent mean \pm S.E. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$.

represented 74% of S_1 -induced H -outflow compared to 38% during S_1 (Table IV S_{max} rates). The stimulation-induced ^3H -outflows were 51.800 ± 2.800 dpm (S_1) and 10.800 ± 2.00 dpm (S_6) ($P < 0.001$ $N = 6$). Accordingly the absolute amount of ^3H NA released by S_1 and S_6 was the same: 5.630 ± 0.670 dpm (S_1) and 4.100 ± 0.900 dpm (S_6) ($P > 0.05$ $N = 6$).

Discussion

The present work provides strong evidence for the view that the ^3H -outflow induced by electrical-field stimulation of isolated rabbit main pulmonary artery preloaded with ^3H NA of neuronal as well as extraneuronal origin.

That NA is released from nerve terminals in rabbit pulmonary artery is already well established (Bevan *et al.* 1969). This vessel is innervated only by postganglionic adrenergic and constriction sympathetic fibres (Bevan and Su 1964, Verity and Bevan 1968).

That amount by which the stimulation-induced ^3H -outflow is reduced by bretylium (by blocking NA-release from adrenergic neurones; Boura and Green 1959, Herring *et al.*

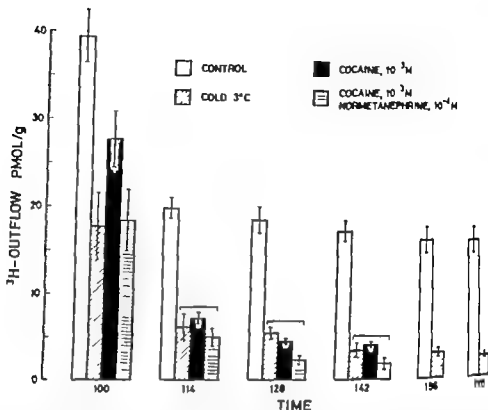
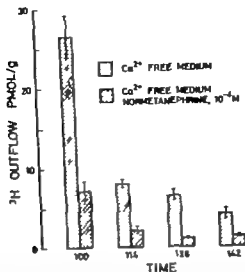


Fig. 3 Effect of cold, cocaine or normetanephrine on electrical-field stimulation induced ³H-outflow from isolated rabbit pulmonary artery preloaded with H NA. Ordinate: Mean stimulation-induced outflow (pmol g⁻¹). Abcissa: Time (min) after commencement of wash-out period. Cocaine (10⁻³ M) plus normetanephrine (10⁻⁴ M) and cold (3°C) was applied 20 min prior to the addition of H NA (10⁻⁴ M). All treatments were maintained during the remainder of the experiment. The ³H-outflows (pmol g⁻¹ 2 min⁻¹) and tissue H-contents (pmol g⁻¹) before S₁ were (mean ± S.E.): 165 ± 16 and 2026 ± 165 (control); 119 ± 16 and 484 ± 46 (cold); 152 ± 19 and 590 ± 67 (cocaine); and 162 ± 16 and 522 ± 96 (cocaine + normetanephrine). Each column is the mean of 4–11 determinations (control) 12, cold 4 cocaine 4 cocaine plus normetanephrine 4). Vertical bars denote ± S.E. No significant difference between cocaine and cocaine plus normetanephrine. P > 0.05, P < 0.01, P < 0.001.

1962), TTX (by blocking axonal action potentials, Narahashi 1974) or omission of Ca²⁺ from the bath fluid (Ca²⁺ is a prerequisite for stimulation-evoked NA-release, Kirpel 1974) is considered proportional to the release of H NA from adrenergic neurones.

The following findings suggest that the H-outflow induced by electrical-field stimulation of the pulmonary artery preloaded with H NA in part represents transmitter release from extraneuronal binding sites. First TTX failed to abolish the stimulation-induced outflow (Table III Fig. 2) in a concentration that blocked the response to transmural stimulation. TTX likewise failed to abolish the stimulation-induced H-outflow from the perfused rabbit carotid artery preloaded with H NA (Allen *et al.* 1973). In contrast, TTX in a lower concentration than we used (10⁻⁶ M) abolished the H NA outflow as well as contractions induced by transmural stimulation of the rabbit pulmonary artery (Su and Bevan 1970). This was also the case for human blood vessels (Sjöström and Gripe 1973, Sjöström and Brundin 1975). Secondly bretylium failed to abolish the stimulation-induced outflow (Fig. 2, Table III) while blocked the contractile tension responses. On the other hand, bretylium consistently



4 Effect of normetanephrine on the Ca^{2+} -sensitive H^+ -outflow induced by electrical-field stimulation of rabbit periaortic artery. Ordinate: Mean stimulation-induced ^3H -outflow (pmol g^{-1}). Abscissa: s (time) after the commencement of the sub-out period. Ca^{2+} -free PBS was used. Normetanephrine added 20 min prior to the incubation with ^3H -NA (10^{-6} M). The passive ^3H -outflows (pmol $\text{g}^{-1} \text{ min}^{-1}$) before S_1 were (mean \pm S.E.): 2.5 ± 1.5 (Ca^{2+} -free) and 20.4 ± 3.6 (Ca^{2+} -free normetanephrine). (These ^3H -contents before S_1 are given in Table III. Each column is the mean of 6–10 determinations \pm S.E. $P < 0.01$ $P < 0.001$.)

NA TV Metabolism of ^3H -NA released spontaneously and by electrical-field stimulation of rabbit isolated periaortic arteries. Artery rings were incubated with ^3H -NA (10^{-6} M) for 45 min. After 100 min wash-out period the rings were stimulated at 100 min (S_1) and at 114 min (S_2).

Stimulus	Percent of total radioactivity											
	NA				DOPEG				OMDA			
	P	P'	S	S _{corr}	P	P'	S	S _{corr}	P	P'	S	S _{corr}
aid stimulation												
S_1	12.8 ± 2.1	14.0 ± 2.3	11.4 ± 1.3	11.0 ± 1.3	13.6 ± 1.0	14.9 ± 0.8	6.3 ± 0.6	3.5 ± 0.5	41.4 ± 3.4	37.3 ± 2.9	64.6 ± 2.5	73.6 ± 3.7
S_2	15.2 ± 2.7	16.9 ± 2.8	25.1 ± 2.2	38.2 ± 2.8	18.0 ± 1.6	17.6 ± 1.6	13.4 ± 1.8	7.7 ± 2.5	42.3 ± 1.6	39.3 ± 1.6	44.1 ± 5.6	38.9 ± 2.9

NA: ^3H -noradrenaline. DOPEG: ^3H -3,4-dihydroxyphenylethyl. OMDA: H-O-methylated and demethylated metabolites.

P and P' represent radioactivity in the next to the last and the last fraction, respectively just prior to stimulation.

S represents the stimulation-induced release of radioactivity in the fraction during stimulation.

S_{corr} represents the stimulation-induced release of radioactivity during S corrected for passive effect (for calculation see Methods).

Values represent mean \pm S.E. (n = 6). The level of significance for the difference between S_1 and S_2 is as indicated: NS: not significant; $P > 0.05$; * $P < 0.01$; ** $P < 0.001$.

abolished both the H NA outflow and the contractile response to transmural stim (Su and Bevan 1970). *Thirdly* Ca^{++} -deprivation failed to abolish the stimulation-induced outflow (Fig. 2, Table II). The discrepancies between our results and those of Su and (1970) with regard to the effect of TTX and bretylium on H-outflow cannot be explained. Su and Bevan (1970) used a superfusion technique while we employed fractional collection-method (Nedergaard and Schrold 1973). It is unlikely that the discrepancies are due to this difference in experimental conditions. Using the superfusion technique we have recently found that bretylium did not abolish the H-outflow (Schrold Nedergaard, unpublished). The different results may possibly be related to the stimulus used and the time at which the effect of adrenergic neurone blocking agents were tested. Su and Bevan (1970) apparently stimulated their preparation several times with train of pulses (1 200 vs. ours 300) before the adrenergic neurone blocking agent was added at the end of the expt. (cf. their Fig. 4). This stimulation procedure may perhaps have released tritium from extraneuronal sites. This possibility gains support from recent finding that when the pulmonary artery ring was first stimulated intensely bretylium markedly reduced the stimulation induced H-outflow (Schrold and Nedergaard unpublished). The failure of bretylium and TTX to abolish stimulation-induced H-outflow may not be due to submaximal treatment. This is supported by the findings that these treatments caused a block of the neurogenic tension response. Furthermore, the H-outflow (g^{-1}) was approximately the same for both these treatments and Ca^{++} -deprivation (indicating that the same maximal effect on H-outflow was achieved).

Fourthly the H-outflows could be evoked by stimulation of artery rings which had been preloaded with H NA in the cold or in the presence of cocaine (Fig. 3). The uptake of adrenergic neurones via the membrane pump is inhibited by cold or by cocaine (Nedergaard and Bevan 1971). Thus, it may be inferred that the H NA was taken up and bound at extraneuronal sites from which it was released by field stimulation.

H NA is taken up and bound by neuronal (uptake-1) as well as by extraneuronal sites such as vascular smooth muscle (uptake 2) and connective tissue (Avakian *et al.* 1968, Nedergaard and Bevan 1971, Powis 1973). When the artery rings were preloaded with H NA in the presence of a specific uptake 2 inhibitor NMN (Iversen 1965) the stimulation-induced H-outflows in Ca^{++} -free PSS were markedly reduced (Fig. 4). This suggests that the stimulation-induced H NA originates in part from uptake 2 binding sites. Direct evidence for the view that the stimulation induced H-outflow from a blood vessel preloaded with H NA originates in part from extraneuronal sites has recently been obtained (Schrold and Nedergaard 1976). When rabbit aortic tunica media, which contains no adrenergic neurones, was preloaded with H NA, field stimulation caused a Ca^{++} -insensitive H-outflow.

The H-outflows and tension responses induced by electrical-field stimulation showed a clear dissociation (Table I, Fig. 1). The stimulation induced H-outflows declined markedly after the first period of stimulation and then remained almost constant. In contrast the concomitant tension responses increased initially and then remained approximately the same. Su and Bevan (1967, 1970) using the superfused rabbit pulmonary artery likewise found that the H-outflow evoked by the initial period of field stimulation was disproportionately great compared to those elicited by subsequent periods of stimulation.

le the tension responses remained constant. On the other hand, Starke *et al.* (1974) using the same preparation found that the H-outflow induced by S_2 was 90% of that induced by S_1 .

A disproportionately great S_2 -induced ^3H -outflow was also found in Ca^{++} -free medium (Fig. 2), in the presence of bretylium (Fig. 2) or TTX (Fig. 2), or under conditions (cold and saline; Fig. 3) where uptake-1 was inhibited during the preloading period with ^3H NA. When the extraneuronal ^3H -outflows (Ca^{++} -insensitive) were subtracted from the corresponding outflows from untreated tissue, the calculated H-outflows were the same in six periods (cf. Fig. 2, column 1 minus column 2). These ^3H -outflows most likely represent release of ^3H -NA from adrenergic neurones. This concept also provides closer correlation between neuronal H-outflows and concomitant tension responses. Additional evidence for this view was obtained by the finding that the absolute amount of H-NA released by S_1 and S_2 was the same. The small increase in the initial tension responses cannot be related to a corresponding increase in ^3H NA release, but must be due to other factors. Since the initial stimulation-induced H-outflow (S_1) represents a disproportionately high amount of tritium from extraneuronal sites compared to subsequent outflows, it is particularly unsuited for studies of release of H-NA from adrenergic neurones. In the light of this, results from studies (Schrold and Nedergaard 1973, 1974) in which S_1 has been used need to be reexamined.

The view that the initial stimulation-induced ^3H -outflow (S_1) is predominantly of extraneuronal origin gains further support from the finding that the distribution of the total H-outflow on ^3H NA and its H-metabolites elicited by S_1 was quite different from that of S_2 (Table IV). This is in contrast to Sz and Bevan (1970), who found an almost equal percentage proportion of H-NA released during S_1 and S_2 . Since these authors likewise found a much greater total H-outflow during S_1 than S_2 , the absolute amount of H-NA released by S_1 must have been greater than that of S_2 . This seems surprising, since Sz and Bevan found that S_1 and S_2 elicited a tension response of equal size.

H-NA and ^3H -DOPEG represented approximately 15% each of the total H-efflux (Table IV). Sz and Bevan (1970) using the superfused rabbit pulmonary artery strip found that ^3H -NA constituted 30% of the total tritium in the superfusate. However this value is in good accordance with ours, since their determination of H-NA included ^3H -DOPEG. A direct comparison of the values found during stimulation seems less relevant, because different stimulation parameters were used.

The mechanism of release of ^3H NA and its metabolites from extraneuronal sites can only be guessed at. It could be due to a local depolarization causing sufficient release of Ca^{++} which in turn triggers the ^3H -outflow. Alternatively it may be likened to the electrophoretic release from an ion exchange resin.

^3H -outflow of extraneuronal origin may possibly also occur in other tissues. Thus, Katz and Kopin (1969) found that field stimulation induced H-outflow from rat atria preloaded with ^3H NA also had a Ca^{++} -insensitive component.

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Experimental Cardiac Hypertrophy and the Synthesis of Poly(A) Containing RNA and of Myocardial Proteins in the Rat The Effect of Digitoxin Treatment

By

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Abstract

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The synthesis of poly(A) containing RNA was increased in heart of non-digitoxinized and digitoxinized rats. After aortic constriction, the increase being of the same degree as that of the RNA lacking this sequence. No differences were found, either in the absence or presence of polyuridylic acid, in the incorporation of radioactivity into protein by cardiac ribosomes isolated from animals treated differently. It was concluded, that after the constriction of the aorta the synthesis of mRNA proceeds at a similar rate as that of the bulk RNA, and that the treatment of the animals with digitoxin does not abolish the stimulus of hypertrophy.

As the heart adapts to work-overload caused by constriction of the aorta, ribonucleic acids (Kohde *et al* 1969 Fanburg *et al* 1968) and protein synthesis (Posner *et al* 1968, Fick *et al* 1971) are stimulated in the myocardium. A positive correlation between the increase in heart mass and both the increase in RNA concentration (Nair *et al* 1969) and the increase in myocardial protein synthesis (Zak *et al* 1971) has been observed. However, treatment of the animal with digitoxin has been shown to prevent the increase in heart mass (Williams *et al* 1965 Turto *et al* 1973) but without marked effect on RNA synthesis (Posner *et al* 1968 Turto *et al* 1973).

After the onset of work-load, the synthesis of ribosomal RNA (rRNA) and transfer RNA (tRNA) increases rapidly but the distribution of the radioactive label in various fractions of RNA (28S, 18S and 4S) has been shown to be the same for the hearts of animals with and without aortic constriction (Fanburg *et al* 1968 Kohde *et al* 1969) Moroz (1967) and Fick *et al* (1971) did not find any evidence for more active ribosomal fractions or for larger portions of ribosomal aggregates as a result of hypertrophy. However, Meerson *et al* (1974) were able to observe an increased ratio of translating poly(A) nontranslating

some monomers and subunits and the authors suggested that the synthesis of mRNA increase to a greater extent than the synthesis of rRNA.

In the present study myocardial RNA synthesis in non-digitalized and digitalized rats with aortic constriction has been studied. Cytoplasmic and nuclear RNA were isolated by the thermal phenol method (Markov *et al.* 1973). Polyadenylic acid (poly(A)) containing A from these two fractions was isolated using oligodeoxythymidylate (oligo(DT))-dye columns. Also the incorporation of radioactivity from aminoacyl-tRNA into products by ribosomes isolated from the hearts of non-digitalized and digitalized rats with and without constriction of aorta was measured.

Material and Methods

In albino rats of the Wistar strain, two months of age and weighing about 200 g, were used. Half of the rats received dehydrin (0.1 mg/100 g b.wt.), dissolved in a mixture of ethanol:glycerol:water (1:1:1), for 6 days each week. The other animals received the same amount of solvent alone. Dehydration started four days before operation.

Constriction of the aorta. Half of the non-digitalized and digitalized animals underwent constriction of abdominal aorta as described earlier (Turto *et al.* 1973). The other half of the animals underwent no operation.

Labelling of RNA. At selected times after the operation the animals were injected i.p. with $\mu\text{Ci/g b.wt.}$ carrier free $^{32}\text{P}(\text{H}_2\text{PO}_4)$ (American Radiochemical Center) diluted into 1 ml of saline. The animals were sacrificed 4 h later and the hearts were removed. The atria and great vessels were cut away and the ribs were removed rapidly in saline, blotted dry and weighed.

Determination of total RNA. Total RNA was determined by the orcinol method (Altmann *et al.* 1945) as described earlier (Turto *et al.* 1973).

Extraction and purification of RNA from heart. For sucrose density gradient analysis, RNA was extracted by the method of Potter and Fastburg (1966). The tissue was minced with scissors in ice-cold 20 mM Tris-HCl buffer (pH 7.2) and homogenized in 20 volumes of the buffer with a teflon-glass Potter-Elvehjem homogenizer. The homogenate was made 0.5% with respect to sodiumdodecylsulphate (SDS) and was mixed with an equal volume of 88% phenol containing 0.1% 8-hydroxyquinoline for 30 min at 4°C. The aqueous phase was re-extracted with phenol, and after adding one tenth volume of 2% sodium acetate (pH 5.2) RNA was precipitated with 2.5 volumes of 95% ethanol at -20°C for 4 h.

DNA was removed from the precipitate by treatment with 20 μg of RNAase free DNAase (Sigma Chemical Co) for 30 min at room temperature in 2 ml of 20 mM Tris-HCl (pH 7.2) containing 20 mM $\beta\text{-SO}$. After adding ice-cold water and SDS to final concentration of 0.5% the mixture was deproteinized with phenol, and RNA was precipitated from the aqueous phase with sodium acetate and ethanol. Purified DNA was removed from the precipitate by 2 precipitations with ethanol (final concentration 1%) at high salt concentration (Peebles *et al.* 1966) and the final precipitate was dissolved in 2% sodium citrate (pH 5.2).

For the preparation of poly(A) containing RNA, cytoplasmic and nuclear RNA were extracted by the thermal phenol method (Markov *et al.* 1973; Georgiev 1967). Heart tissue, 5 to 7 g, was homogenized in 10 volumes of ice-cold 0.14 M NaCl and an equal volume of water-saturated phenol (pH 6.2) containing 1% 8-hydroxyquinoline was added. After shaking for 15 min at 4°C the phases were separated by centrifugation. The aqueous phase was deproteinized and the interphase layer was re-extracted at 4°C. The protein phase was discarded and the interphase was extracted with 0.14 M NaCl and phenol at 75°C. The aqueous phases were deproteinized two times in the presence of 1% SDS, once with phenol:chloroform (1:1), once with chloroform and finally the RNA was precipitated with 3 volumes of ethanol containing 2% sodium acetate at -20°C overnight. DNAase treatment was carried out as described before. For sucrose density gradient analysis of RNA. About 200 μg of RNA in 0.2 ml of 2% sodium acetate was spread on 5 ml of 5-20% linear sucrose gradient, made in 2% sodium acetate (pH 5.2) (Markov *et al.* 1964). The tubes were centrifuged in a Spinco SW 50 rotor at 24000 rpm for 14 h. Fractions (25-27 fractions) were collected by puncturing the bottom of the tube. Optical density was measured at 260 nm.

with the Zeiss M4 QIII spectrophotometer after adding distilled water. Absorbancy of blank fraction subtracted from each reading. Direction of sedimentation in figures is from right to left.

RNA was precipitated with 10% trichloroacetic acid (TCA) after adding 100 μ g of carrier yeast. The precipitate was collected on Whatman glass-fiber filter, washed with 50 ml of cold 5% TCA and of 80% ethanol. The filter was dried, dissolved in 0.5 ml of Soluene 100 (Packard) and counted in Gel (Packard).

Oligo(dT) fractionation of RNA. RNA containing poly(A) sequences is known to bind to poly(A) by base pairing (Edmonds *et al.* 1969). No more than 30 A_{260} units of RNA were dissolved in 0.01 M HCl buffer (pH 7.5) containing 0.4 M NaCl and was applied in a 1.0 ml oligo(dT)-cellulose column (Collaborative Research) previously washed with the application buffer. The non-absorbed fraction was collected and the column was washed with the application buffer. The bound fraction was eluted with 0.01 M Tris HCl buffer (Aviv *et al.* 1972) and the absorbancy at 260 nm was measured. After the elution of carrier RNA the fractions were precipitated with 10% TCA, collected on glass fiber filters and prepared for radioactive counting as described above.

Preparation of ATP from heart tissue. Tissue ATP was prepared essentially as described by Fiske (1968). Potato apyrase (Sigma), 0.5 mg/ml, was used to hydrolyze tissue ATP and the resulting AMP separated by Dowex 1-formate chromatography (Bergqvist *et al.* 1954). The purity of AMP was checked by paper electrophoresis (Klouwen 1962). AMP prepared in this manner represents the AMP from tissue ATP, since the amount of free AMP in tissue is negligible (Threlkell 1957).

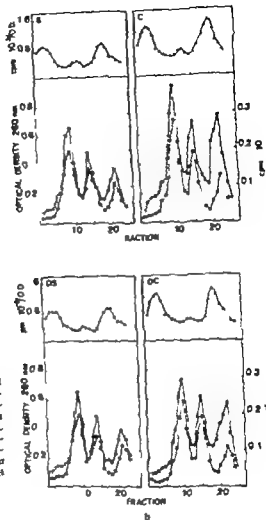
The specific activity of precursor ATP (determined as AMP) was used to correct RNA labeling. Percent stimulation in RNA labeling was calculated by subtracting the RNA:AMP ratio of sham-operated animals from that of the animals with constriction of the aorta and dividing the result by the RNA:AMP ratio of sham-operated animals and multiplying by 100. The units for RNA and AMP are cpm.

Preparation of heart muscle ribosomes. For the preparation of ribosomes (Castles *et al.* 1972) heart tissue was finely minced with a razor blade and homogenized in a buffer containing 0.90 M Tris (pH 7.6), 12.5 mM $MgCl_2$, 250 mM KCl and 250 mM sucrose with a teflon-glass Potter-Elvehjem homogenizer. After centrifugation at 13 000 g for 10 min the pellet was re-extracted with the homogenization medium and the combined supernatants were centrifuged at 78 000 g for 2 h. The resulting pellet was resuspended in medium A having the same composition as that of the homogenization medium except the concentration of KCl was reduced to 80 mM. The suspension was treated with Triton X 100 and deoxycholate (final concentrations 0.5% and 1%, respectively). The precipitate of myosin was removed by centrifugation for 15 min at 13 000 g and 7 ml of the supernatant was layered over 5 ml of 1 M sucrose in medium A. The ribosomes were pelleted by centrifugation at 105 000 g for 4 h.

For sucrose density gradient analysis, ribosomes were loaded on the top of a 15 to 30% linear sucrose gradient prepared in medium A, and the tubes were centrifuged for 90 min at 37 500 rpm in a Spinco rotor. Twenty-five fractions were collected and the optical density at 260 nm was determined.

Preparation of labelled aminoacyl-tRNA. Aminoacyl-tRNA was prepared using tRNA extracted from rat liver (Deutscher 1974) and the enzyme fraction prepared from rat liver (Wigle 1973). The tRNA was charged with L-[4,5- 3H]leucine, L-[^{14}C]phenylalanine and 19 additional amino acids as described by Wigle (1973). The specific radioactivity of labelled aminoacyl-tRNA was calculated from its absorbance at 260 nm (μ g of RNA/ml = $OD_{260} \times 0.0221$) and its radioactivity. The supernatant protein (transferase preparation) was prepared from the 78 000 g supernatant of rat liver homogenate (Castles *et al.* 1972).

Protein synthetic activity of ribosome in vitro. The ratio of A_{260}/A_{280} of the ribosomal preparations varied from 1.79 to 1.87 indicating the purity of the ribosomes. RNA concentration in ribosome preparations was determined by modification (Wool *et al.* 1967) of the method of Fleck *et al.* (1962). The ribosomes were diluted with medium A to the concentration required. The assay of protein synthesis of ribosomes was carried out in a total volume of 1.0 ml containing 30 μ mol of Tris-HCl (pH 7.8), 8 mM KCl, 12.5 μ mol of $MgCl_2$, 10 μ mol of 2-mercaptoethanol, 1 μ mol of ATP, 0.4 μ mol of GTP, 10 μ mol of creatine phosphate, 0.1 mg of creatine phosphokinase, 100 μ g of tRNA labelled with [3H]leucine, [^{14}C]phenylalanine and 19 additional amino acids, and 2.5 mg of a permutant protein. The amino acid polyuridylic acid, when present, was 100 μ g. The reaction was initiated by adding 0.1 ml of ribosome suspension. The incubation was for 30 min at 37°C in a water bath shaker. When any of these conditions were changed, the details of the change made are given in the legends to the figures. The reaction was terminated by adding 0.1 ml of 3 M KOH and the incubation was continued for 30 min. The samples were prepared for counting (Castles *et al.* 1972), dissolved in Soluene 100 and counted in Fast



1. Patterns of labelling of myocardial tissue two days after the operation in non-digitalized rats with (C) and without (S) constriction of aorta, and digitalized rats with (D) and without (DS) constriction of aorta. Circles = counts/min, solid circles = optical density, solid triangles = specific radioactivity (obtained by dividing the counts at fraction by the optical density at that fraction).

Results

Constriction of the aorta resulted in an increase in the ratio of heart weight to body weight, the increase being about 10% at 2 days and about 19% at 4 days after constriction of the aorta in non-digitalized animals. In digitalized animals the increase was about 4% at 2 days and 8% at 4 days, respectively.

Also, an increase in the myocardial RNA concentration was observed 2 days after the constriction of the aorta, the values (mg/g wet weight of tissue, mean \pm S.D.) being 49 ± 0.18 in sham-operated, 2.82 ± 0.21 in animals with constriction of the aorta ($p < 0.02$), 34 ± 0.17 in digitalized sham-operated and 2.58 ± 0.18 in digitalized animals with constriction of the aorta ($p < 0.05$). At 4 days the values were 2.42 ± 0.16 , 3.03 ± 0.31 ($p < 0.005$), 3.8 ± 0.20 and 2.84 ± 0.24 ($p < 0.01$), respectively.

TABLE I Labelling of RNA. Non-digitalized and digitalized (D) animals underwent either sham-op (S) or constriction of aorta (C). The specific activity of RNA was obtained by dividing the count/min by the sum of optical densities of the entire sucrose gradient (Fig. 1)

Group	Hypertrophy %	RNA cpm/OD	AMP cpm/OD	% increase in sp. activity of RNA
S		431	2 200	
C	9.6	719	2 820	30.1
DS		444	2 210	
DC	3.5	649	2 700	19.6

Fig. 1 demonstrates the labelling pattern of cardiac RNA two days after the op. RNA is separated into three peaks on a 5 to 20% sucrose density gradient, which has been shown to correspond in sedimentation characteristics (28S, 18S and 4S) to the species from liver (Posner *et al.* 1966). Any marked changes could not be seen in the labelling pattern of RNA extracted from the hearts of non-digitalized and digitalized animals with and without constriction of aorta. However, after correcting the specific activity of AMP, the extent of labelling of total RNA after aortic constriction was 30% greater in non-digitalized animals and 20% greater in digitalized animals compared to the values obtained for the respective controls (Table I).

Phenol extraction and oligo(dT)-cellulose fractionation of RNA Two types of RNA were obtained by the thermal phenol method. The RNA extracted at 4°C contained the 28S and 4S peaks, typical for the cytoplasmic RNA, whereas the radioactivity of the RNA extracted at 75°C was rather evenly distributed throughout the sucrose gradient and was considered to be of nuclear origin (Fig. 2) (Georgiev 1967; Markov *et al.* 1973). The incorporation of radioactivity into the oligo(dT) unbound and bound fractions of both cytoplasmic and nuclear RNA was linear for at least 6 h. Also the increase in the labelling of AMP was linear for at least six hours following the injection of the label (Fig. 3). The specific radioactivity of the nuclear RNA was about two times the value for the cytoplasmic RNA and the specific radioactivity of RNA bound to oligo(dT) was about twice that for the unbound fraction (Table II).

The labelling of cytoplasmic and nuclear RNA containing poly(A) (the fraction bound to oligo(dT)-cellulose) was increased by about 60% at 2 days and about 44% at 4 days after constriction of the aorta. In digitalized animals the increase was 44% and

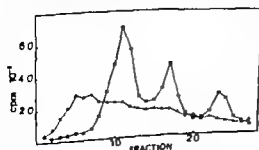
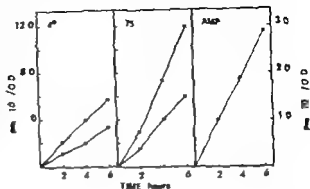


Fig. 1. The pattern of labelling of myocardial RNA extracted at 4°C (○) and 75°C (Δ). The animals were injected with 1 mCi of [³²P]UTP 4 h later.

3 Time course of labeling of portion of tissue ATP and topoisomerase (extracted at 4°C) nuclear (extracted at 75°C) fractionated by the oligo(dT) cellulose columns (□) fraction RNA that not bound to oligo(dT), (■) poly(A) containing



pectively. The increase in the specific activity of RNA that was not bound to oligo(dT) was of the same degree in both thermal fractions (Table II).

Protein synthesis by cardiac ribosomes *in vitro*. Sucrose density gradient analysis of these ribosomes of non-distalized and distalized animals with aortic constriction did not show any substantial shift to larger polyosomes if compared with the respective controls (Fig. 4). The assay of ribosomal activity *in vitro* showed that [³H]leucine was incorporated into protein rapidly for the first 10 min and thereafter the rate declined until after 30 min further synthesis occurred (Fig. 5 on the left).

The incorporation of radioactivity from [³H]leucyl-tRNA into protein was directly proportional to the concentration of ribosomes (at least up to 60 µg of ribosomal RNA/ml (Fig. 5 in the middle). The saturating amount of aminoacyl-tRNA for 30 µg of ribosomal RNA was about 200 µg (Fig. 5, on the right). In the experiments, the amount of aminoacyl-tRNA (100 µg) was not saturating, but since the protein synthesis was linearly proportional to the concentration of ribosomes, it was not considered limiting.

TABLE II. The effect of constriction of aorta and digoxin treatment on the labeling of RNA fractionated by the oligo(dT) cellulose columns. The groups are marked as in Table I. The numbers in parentheses show the per cent increase in the specific radioactivity of RNA over the respective control.

Group	Hypertrophy	AMP cpm/OD	RNA extracted at 4°C		RNA extracted at 75°C	
			Not bound to oligo(dT) cpm/OD	Bound to oligo(dT) cpm/OD	Not bound to oligo(dT) cpm/OD	Bound to oligo(dT) cpm/OD
14 days after operation						
		210	293	590	412	1160
DS	12	2930	995 (53.1)	1153 (58.4)	833 (52.3)	2572 (66.5)
DC	5	2043	235	496	381	1232
		3100	540 (40.5)	1033 (41.7)	796 (39.5)	2700 (46.1)
20 days after operation						
		2520	206	394	413	743
C	20	3570	571 (26.7)	721 (28.3)	720 (22.8)	1316 (26.8)
DS		2260	210	376	386	658
DC	8	3300	347 (18.0)	664 (20.6)	637 (13.3)	1092 (11.7)

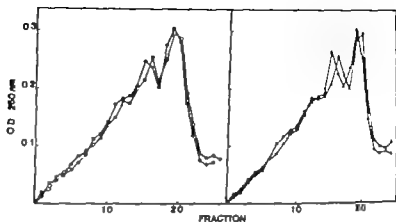


Fig. 4. Sucrose density gradient distribution of ribosomes isolated from the hearts of non-digitalis without (O) and with (●) constriction of the aorta, and of digitalized rats without (Δ) and with (▲) constriction of the aorta.

The ribosomes isolated from the hearts of different groups of animals had the same course of incorporation of radioactivity into protein, they had the same optimum concentration for aminoacyl-tRNA and the incorporation of radioactivity into protein was directly proportional to the concentration of ribosomes. The addition of polysaccharide into the reaction mixture increased the incorporation of radioactivity from

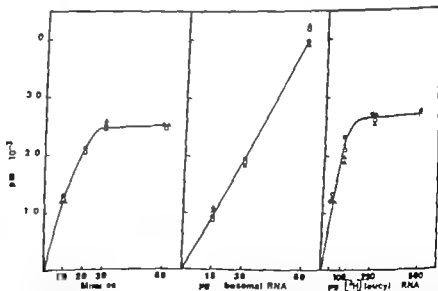


Fig. 5. Time course of incorporation of radioactivity from aminoacyl-tRNA into protein by cardiac ribosomes. The ribosomes, containing 30 μ g of ribosomal RNA, were incubated with 100 μ M [3 H]leucyl-tRNA (59 635 cpm) for different time periods (on the left). The effect of concentration of ribosomes on the incorporation of radioactivity into protein. Different concentrations of ribosomes incubated with 100 μ g of [3 H]leucyl-tRNA (59 635 cpm) for 30 min (in the middle). The effect of concentration of aminoacyl-tRNA on the incorporation of radioactivity into protein by cardiac ribosomes. Ribosomes containing 30 μ g of ribosomal RNA, were incubated with different concentrations of [3 H]leucyl-tRNA for 30 min (on the right). Ribosomes were isolated from the heart of non-digitalis without (O) and with (●) constriction of the aorta, and of digitalized rats without (Δ) and with (▲) constriction of the aorta.

- III. Incorporation of radioactivity from sarkosyl-³H-leucyl-tRNA into protein by ribosomes isolated four days after the operation from the heart tissue of non-digitalized and digitalized animals with and without constriction of the aorta. Ribosomes, containing 30 µg of RNA were incubated with 100 µM of [³H]leucyl-tRNA (39 635 cpm) or [¹⁴C]phenylalanyl-tRNA (34 346 cpm) as described in Material and Methods. The concentration of poly(U), when present, was 100 µg/ml.

no of ribosomes	Incubation time, min	Hypertrophy %	Incorporation, cpm		
			[³ H] leucyl-tRNA	[¹⁴ C] phenylalanyl-tRNA	
				- poly(U)	+ poly(U)
	10			857 ± 47	2 900 ± 202
	30		2 163 ± 175	1 404 ± 40	5 055 ± 144
	10			924 ± 63	2 822 ± 173
	30	11	2 230 ± 229	1 426 ± 61	5 208 ± 249
	10			840 ± 72	2 856 ± 145
	30		1 974 ± 189	1 379 ± 60	5 157 ± 253
	10			900 ± 30	2 686 ± 226
	30	7	2 135 ± 326	1 417 ± 69	5 101 ± 173

Mean ± S.D. of 4 determinations.

Mean ± S.D. of 3 parallel determinations.

myalanyl-tRNA into protein about threefold. However no substantial difference was found in the incorporation of radioactivity into protein by ribosomes isolated from different parts of animals (Table III).

Discussion

Several studies have demonstrated an early increase in the synthesis of myocardial RNA following the constriction of aorta and leading later to an increased RNA content of the heart (McCrone 1969, Fanburg *et al.* 1968, Koldo *et al.* 1969, Turtio *et al.* 1973). In the present study a marked increase in the labelling of RNA was observed 2 days after the operation. The specific activity of α-phosphate of tissue ATP was used to correct the labelling of RNA. The adequacy with which this type of correction reflects precursor specific activity depends upon an assumption of the absence of compartmentalization of the precursor in the tissue and similar rates for the incorporation of other nucleotides into RNA as that for the deoxyribose nucleotide.

Subsequent to the constriction of the aorta a generalized stimulation in the labelling of all species of RNA throughout the entire myocardial gradient was found. No selective increase in the labelling was found in any particular area of the gradient. The results presented here are consistent with those of Fanburg *et al.* (1968) and Koldo *et al.* (1969).

The earlier studies (Fanburg *et al.* 1968, Koldo *et al.* 1969) did not reveal any evidence for an increased synthesis of mRNA in heart tissue undergoing hypertrophy as measured by the base composition analysis. McCrone *et al.* (1974) have suggested an increased synthesis of mRNA as a possible explanation for the finding of an increased ratio of translating to non-translating ribosomes in the hypertrophic heart. In the present study an approach was made to study mRNA synthesis by isolating poly(A) containing RNA by the oligo(dT)-cellulose column.

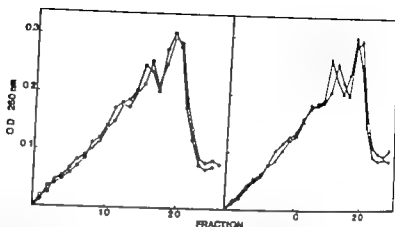


Fig. 4. Sucrose density gradient distribution of ribosomes isolated from the hearts of non-digitalized without (O) and with (●) constriction of the aorta, and of digitalized rats without (Δ) and with (▲) constriction of the aorta.

The ribosomes isolated from the hearts of different groups of animals had the same course of incorporation of radioactivity into protein. They had the same optimum concentration for aminoacyl-tRNA and the incorporation of radioactivity into protein was directly proportional to the concentration of ribosomes. The addition of polyacid into the reaction mixture increased the incorporation of radioactivity from

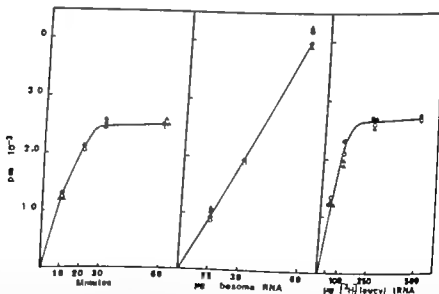


Fig. 5. Time course of incorporation of radioactivity from aminoacyl-tRNA into protein by ribosomes. The ribosomes, containing 30 μ g of ribosomal RNA, were incubated with 100 μ g of 3 H-leucyl-tRNA (59 635 cpm) for different time periods (on the left). The effect of concentration of ribosomes on the incorporation of radioactivity into protein. Different concentrations of ribosomes, incubated with 100 μ g of 3 H-leucyl-tRNA (59 635 cpm) for 30 min (in the middle). The effect of concentration of aminoacyl-tRNA on the incorporation of radioactivity into protein by cardiac ribosomes. Ribosomes containing 30 μ g of ribosomal RNA were incubated with different concentrations of 3 H-leucyl-tRNA for 30 min (on the right). Ribosomes were isolated from the heart of non-digitalized without (O) and with (●) constriction of the aorta, and of digitalized rats without (Δ) and with (▲) constriction of the aorta.

hypertrophy (Williams *et al.* 1965 Turto *et al.* 1973) and myocardial protein synthesis (Turto *et al.* 1973) it has a smaller effect on RNA synthesis (Posner *et al.* 1968, Turto *et al.* 1973) and on the increase in the activity of prolyl hydroxylase (Turto 1977). In the present treatment of the animals with digitoxin did not have effect on the incorporation of activity from aminoacyl-tRNA into proteins by cardiac ribosomes *in vitro*. The effect of digitoxin treatment on the soluble factors in protein synthesis *in vitro* remains unaltered, as results of Moroz (1967) suggest a lack of dependence upon soluble factors, prepared from different sources, in the changes of protein synthesis in cardiac hypertrophy.

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A marked increase in the labelling of cytoplasmic and nuclear RNA was found in the heart of non-digitalized and digitalized animals after the constriction of aorta. The increase in the specific radioactivity of RNA that was not bound to oligo(dT) was of the same degree as that of the poly(A) containing RNA. Due to the difficulties inherent in a gentle homogenization of the heart tissue, some contamination of the cytoplasmic RNA with nuclear RNA would be expected (Markov *et al* 1973). The variation in the amount of poly(A) containing RNA (from 0.5 to 2% of the total RNA) may be due to its binding to proteins during extraction (Braverman *et al* 1972) or due to a contamination with a Mg²⁺ dependent RNase, capable to cleave the poly(A) segment (Rosenfeldt *et al* 1972). In RNA preparations are fractionated by a poly(A) selection procedure, the template acid for protein synthesis has been found to remain associated with the poly(A) containing molecules (Morrison *et al* 1972, Kazazian *et al* 1973) providing evidence for the identification of these molecules as mRNA. From the earlier studies (Fanburg *et al* 1969, Zak *et al* 1971) and the data presented here it may be concluded that there is an enhanced synthesis of mRNA as well as of rRNA and tRNA as a result of aortic constriction. Evidence of increased synthesis of mRNA in the overloaded heart *in vitro* has been reported by Schnitzler *et al* (1968). However, the changes in RNA synthesis may reflect changes primarily in fibroblastic cells, since autoradiographic studies show a predominant labelling of RNA in fibroblastic cells of the muscle tissue (Morkin 1971, Jablonski *et al* 1973).

Contradictory results have appeared regarding protein synthesis in the overloaded heart. Moroz (1967) has demonstrated that ribosomes isolated from hypertrophic hearts are not active in protein synthesis, as measured in an *in vitro* amino acid incorporating system. This difference can entirely be explained by the higher content of ribosomes in microsome fraction isolated from hypertrophic heart. No evidence was found of a larger proportion of ribosomal aggregates. Schreiber *et al* (1968) have found an increased incorporation of radioactivity into proteins by ribosomes isolated from hearts subjected to pressure overload *in vitro* without evidence of larger ribosomal aggregates. Hjalmarsson *et al* (1972) have reported an increase in protein synthesis in association with increased levels of polyribosomes in overloaded perfused hearts. Zak *et al* (1971) did not observe difference in the relative amount of polyribosomes and in the ribosomal activity measured in an *in vitro* amino acid incorporating system between the animals with aortic constriction and controls.

The present results are consistent with those of Zak *et al* (1971). No evidence was found of a larger proportion of ribosomal aggregates or of an increased incorporation of radioactivity from aminoacyl-tRNA into protein by ribosomes isolated from hypertrophic hearts. Zak *et al* (1971) have also found an increase in the labelling and relative amount of membrane-bound ribosomes whereas the labelling of free ribosomes decreases during development of cardiac hypertrophy. However, it is unclear if the increase in the amount and the activity of the membrane-bound ribosomes is due to the increase in the rough endoplasmic reticulum in the muscle cells, or due to the increase in the population of fibroblastic cells, which can make up 75% of the total cell number but only a small percentage of the heart volume (Grove *et al* 1969).

trophy (Williams *et al.* 1965, Turto *et al.* 1973) and myocardial protein synthesis *et al.* 1973) it has a smaller effect on RNA synthesis (Posner *et al.* 1968, Turto *et al.* and on the increase in the activity of prolyl hydroxylase (Turto 1977). In the present treatment of the animals with digitoxin did not have effect on the incorporation of activity from aminoacyl-tRNA into proteins by cardiac ribosomes *in vitro*. The effect of digitoxin treatment on the soluble factors in protein synthesis *in vivo* remains unsolved, the results of Moroz (1967) suggest a lack of dependence upon soluble factors, prepared from different sources, in the changes of protein synthesis in cardiac hypertrophy.

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Relationship between Sarcomere Length and Active Force in Rabbit Papillary Muscle¹

By

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Abstract

WÖRFLART B., A. F. GRIMM and K. A. P. EDMAN. Relationship between sarcomere length and active force in rabbit papillary muscle. *Acta physiol. scand.* 1977 101 155-164

The peak twitch force (stimulation frequency 0.5/s; 29.5-30.5°C) as correlated with sarcomere length in isolated papillary muscles of the rabbit. Sarcomere length was measured from photographic kymographs (1.5 ms exposure time) performed at rest between contractions and at the time of isometric twitch force. The sarcomere length at rest as found to be relatively uniform throughout the preparation and to be linearly related to the overall muscle length within the range $L_{\text{mean}} = 0.85 L_{\text{max}}$. The distribution of sarcomere lengths increased considerably as the muscle went from rest to activity. Studies of the shortening showed different degrees of shortening (or elongation) of individual segments along the length of the preparation. The mean resting sarcomere length at L_{max} (the optimum sarcomere length for production) as $2.44 \pm 0.01 \mu\text{m}$ (grand mean \pm S.E., 7 muscles). The mean active sarcomere length was $2.29 \pm 0.04 \mu\text{m}$. Active force declined steeply as the muscle length as reduced below L_{max} . At resting sarcomere length of 2.0 μm , active force was approximately 1/3 of the maximum. The small differences between the length-tension relationships in myocardium (twitch responses) and skeletal muscle (tetanic contractions) are discussed on the basis of length dependency of the activation process in cardiac muscle.

A characteristic relationship between tetanic force and sarcomere length has previously been defined in single skeletal muscle fibres of the frog (Gordon, Huxley and Julian 1966, Julian 1966). Several attempts have been made to investigate whether a similar relationship exists between peak twitch force and sarcomere length in mammalian myocardium. Studies of this kind (Sonnenblick, Spiro, Cottrill 1963, Grimm and Whitehorn 1963, Grimm *et al.* 1970) are based on sarcomere length determinations on chemically fixed papillary muscles, and the results were therefore most likely complicated by length changes of the sarcomeres induced by the histological procedure itself. Furthermore, as the papillary muscle preparation contains considerable series compliance (e.g. Sonnenblick 1967, Edman and Nilsson 1968, 1969, Nilsson 1972, Julian and Sollins 1975, Krueger and Pollack 1975), which varies

¹The study was performed during substantial leave of absence (June, 1974 - June, 1975) given to A. F. G. by the College of Dentistry and Medicine, University of Illinois at the Medical Center, Chicago, Illinois, U.S.A.

substantially with the techniques used for dissection and mounting, these earlier experiments did not provide definite information as to the relationship between contractile force and sarcomere length of the active myocardium.

Winegrad (1974) and Pollack and Huntsman (1974) have demonstrated the poor value of microscopically examining living isolated cardiac muscle. A resting sarcomere length of 2.35 ± 0.02 (S.E.) μm was found by Krueger and Pollack (1975) at optimum for force production (L_{max}) in rat papillary muscles. Substantial sarcomere shortening occurred during activity along with stretching at the ends of their preparation where damage was probable. Julian and Sollins (1975) studying the sarcomere length-tension relationship in living papillary muscles from small rats, also found considerable sarcomere shortening during contraction. The active sarcomere length-tension relationship derived by the latter authors, however, was shifted to much shorter sarcomere lengths than that reported by Krueger and Pollack (1975) and exhibited a striking similarity with the isometric tension curve obtained for skeletal muscle.

The present investigation is an extension of our previous work (Grimm and Wohlfart 1976) which established a resting sarcomere length at L_{max} of 2.38 ± 0.004 (S.E.) μm in the isolated papillary muscle of the rat. In the present study resting sarcomere lengths were determined along the ascending limb of the length-tension curve in isolated papillary muscles of the rabbit. In addition active sarcomere lengths were determined at the peak of the force-tension twitch. The results of this study suggest that active force of a papillary muscle is more closely related to resting sarcomere length than to sarcomere length recorded during activity.

Methods

Papillary muscles were dissected from the right heart ventricle of young (0.5–0.8 kg) rabbits. The animals were heparinized before their killing and exsanguination. The heart was removed within 2 minutes of the death of the animal and placed in an oxygenated bathing solution at about 35°C. The right ventricle was opened and the dissection was carried out under a Zeiss stereo III microscope at 10 \times magnification. A 6–7 mg loop of platinum wire (diameter 0.23 mm) was tied (braided silk thread, Ethicon 6/0) to the end of a selected papillary muscle *in situ* close to the myotendinous junction. The muscle was then removed from the heart together with the piece of the ventricular wall to which it is attached. The muscle was held at a slightly stretched length in the dissection trough by means of thin needles placed through the tendon loop and the ventricular wall block. The latter was trimmed to a size slightly wider than that of the muscle and a U-shaped loop of platinum wire was tied to it. Care was taken not to touch the muscle between its insertions. The muscles used had a nearly cylindrical shape over approximately 90% of their length. The muscle widened towards the base and tapered towards the tendinous end. Muscles that had the appropriate shape and were thin enough to provide a clear sarcomere pattern were found in about one out of three animals. The thickness of the muscles was 0.3–0.5 mm in the middle of the preparation and the overall length of the muscles at L_{max} (the length for maximum force production) was 2.0–5.5 mm.

Muscle chamber mounting and temperature. The muscle was mounted horizontally in a chamber between a force transducer and glass hook. The chamber was continuously perfused (5 ml/min) with a bathing solution that had been prewarmed to 30°C and was equilibrated with a mixture of 95% O_2 and 5% CO_2 immediately before entering the trough. The bath temperature was 29.5–30.5°C in the different experiments and did not vary by more than $\pm 0.2^\circ\text{C}$ throughout the experiment. The overall length of the muscle was adjusted by means of a micrometer screw which controlled the position of the force transducer. The apparatus has been previously described (Edman and Johansson 1976).

Tension recording. Tension was recorded by means of a strain gauge transducer (compliance 1.4 μm) as described previously (Edman and Johansson 1976). The tension signal was recorded on a Grass polygraph ink writer. The tension was also displayed on a Tektronix 502 A oscilloscope and photographed on film.

muscle. The muscle was stimulated at frequency of 0.5/s by passing current between two platinum electrodes that were placed 1.7 mm apart on either side of the muscle. A unidirectional pulse (2 ms ion, 150% of threshold) was used.

sarcomere. The bathing solution had the following composition (mM): NaCl 100, KCl 5, MgSO₄ 1.5, 2.5 NaHCO₃, 2.5, NaH₂PO₄ 1.5, Na acetate 20, glucose 10, insulin 2 U/l, pH 7.45. All chemicals of analytical grade and dissolved in deaerated, double-distilled water.

measurements of sarcomere length. Light from 100 W microscope lamp (Zeiss) was passed through the excitation. A stereoscopic microscope tube provided with water immersion objective (Zeiss 40 X, N.A. and Zeiss 20 X ocular lens) are used for inspection of the sarcomere pattern. For photographic viewing the microscope was fitted with an extension tube provided with side viewer and 35 mm Prontar or Kodak Tri-X film as used with an exposure time of 1.5 ms. The degree of magnification on the film (40 X) as determined by photographing stage micrometer scale submerged in the bathing solution. Regions of the preparation could be explored by moving the bath on the microscope stage. Clear sarcomere patterns are obtained by focussing close to the upper surface of the muscle; the most distinct patterns found along the edges of the preparation. Pictures were taken in the fully relaxed state between contractions and also during activity. In the latter case the exposure is timed to occur while the contractile force above 54% of peak isometric twitch amplitude. Measurements of the sarcomere length were made on the film with Nikon model 6 C profile projector at 20 X magnification. A row of ten sarcomeres measured and mean value of the sarcomere length obtained. A minimum of 5 measurements were made at each sarcomere length.

Measurements of muscle segments and of overall muscle length. The overall muscle length as measured from the points of attachment of the platinum loops. Such measurements were made at 10 X magnification on the Nikon comparator from photographic record of the muscle adjusted to L_{max} . Changes in muscle length were determined from the setting of the micrometer screw which controlled the position of the force sensor.

Some experiments segmental length changes were studied as the muscle went from rest to full isometric tetanus. For the purpose markers of silk filaments (20 μ m thick and 200-300 μ m long) are placed on muscle surface. Photographic records (see above) of the muscle with its natural and artificial markers taken in the relaxed state between contractions and at the time of peak isometric force. Measurements of the distances between the markers were carried out on the Nikon comparator.

Experimental procedure. After being mounted the muscle was stimulated to contract at frequency of about 1/s before the actual experiment was started. L_{max} was determined by using the following order. The muscle was stretched in steps of 0.05 mm (approximately 2% of the muscle length) until maximum active force was recorded and further increment in length caused reduction of active force. Series of the sarcomere patterns were taken from the peak twitch tension had attained steady-state level. A muscle length as then reduced to give approximately 85, 70, 55, and 40% of optimum peak twitch tension and the sarcomere patterns were photographed at each length. After each length change the muscle was rested to its optimum length and new microphotographs were taken. The sarcomere length measured at L_{max} did not differ significantly (at the 5% level) during the course of an experiment. At the end of each experiment sarcomere length recordings were also made after the muscle had been stretched beyond L_{max} to produce approximately 90% of optimum active force.

Results

Sarcomere length in resting muscle

Photographic records of the sarcomere pattern from three different regions of a papillary muscle between contractions are illustrated in Fig. 1. Measurements from such pictures in a Nikon comparator at 20 X magnification showed that the resting sarcomere lengths at optimum length for force production, L_{max} , was relatively uniform in different regions of the preparation. It should be pointed out that some regions of the muscle, usually along the midline and near the base, did not exhibit clear sarcomere patterns due to the thickness of the preparation and thus could not be judged as to the uniformity of sarcomere length. However measurements from homogenized fixed rat papillary muscles (Grinn and Wohlfart 1974) demonstrated uniformity between the periphery and the core of the preparation.

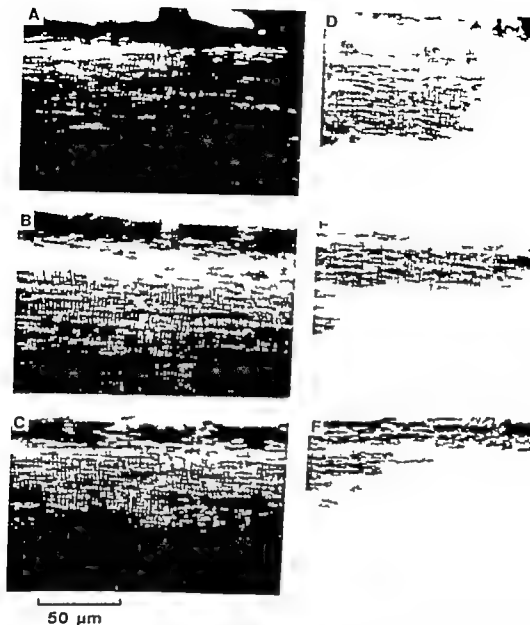


Fig. 1. Sarcomere patterns from three different regions of papillary muscle in the relaxed state (left panel, A, B, C) and at the peak of isometric contraction (right panel, D, E, F) pictures, 1.5 ms exposure time. A, D are close to the tendon while B, E and C, F are from segments in the middle portion of the preparation.

In Table I the resting sarcomere lengths at L_{max} (mean \pm S.D.) are presented for papillary muscles from each of 7 animals. Measurements (usually 5) were made each time the resting length was reestablished at L_{max} after the muscle had been studied at a shorter length. Since the sarcomere length determinations were not statistically different between the determinations at L_{max} , and no muscle slippage could be demonstrated, these mean values per muscle were pooled. As seen in Table I the mean resting sarcomere length at the beginning of the descending limb of the length-tension curve (just before a decrease in force production

1. Sarcomere lengths (SL , μm) at optimum muscle length (L_{max}) at rest and peak activity. Number of measurements in each individual muscle is given without parenthesis.

Rest Mean $SL \pm S.D.$	Activity Mean $SL \pm S.D.$	Ratio SL_a/SL_r
2.43 ± 0.06 (27)	2.34 ± 0.08 (24)	0.96
2.51 ± 0.05 (20)	2.23 ± 0.09 (20)	0.88
2.47 ± 0.06 (5)	2.28 ± 0.08 (5)	0.94
2.45 ± 0.08 (20)	2.29 ± 0.09 (20)	0.93
2.40 ± 0.06 (30)	2.10 ± 0.15 (30)	0.88
2.47 ± 0.05 (25)	2.38 ± 0.11 (25)	0.96
2.41 ± 0.05 (25)	2.44 ± 0.10 (31)	1.01
mean 2.44 ± 0.01 (S.E.)	2.29 ± 0.04 (S.E.)	0.94

rest, see Methods) 2.44 ± 0.01 (S.E.). This value is the grand mean of the mean values in 7 reported muscles without any weighting (or the number of (individual) measurements). Fig. 2 illustrates the relation between overall muscle length and sarcomere length at rest determined in 5 muscles. As indicated by the regression line drawn from the data ($n=217$), there were proportional changes in sarcomere length and overall muscle length. Thus, for a percental change in muscle length (within the range $L_{max} - 0.85 L_{max}$) almost the same change in sarcomere length was obtained. At approximately 85% L_{max} corrugations of the muscle surface and waviness of the myofibrils could be observed microscopically. corrugations of the surface of the muscle were irregular the distance between the folds times the sarcomere length.

Fig. 3 shows the relationship between peak twitch tension and resting sarcomere length papillary muscles. As can be seen there is a relatively moderate scatter of the data points used from different experiments. There was a steep decline in tension as the sarcomere was reduced below $2.4 \mu m$. A 50% decrease in peak twitch force was obtained by resting sarcomere length to $2.05-2.25 \mu m$ in the various experiments.

length in active muscle

Sarcomere length during activity was measured from photographic records taken at the end of isometric peak twitch force (see Methods). Whereas the sarcomere pattern at rest is quite uniform, there was a considerable variation of the sarcomere length in different parts of the muscle during contraction. Furthermore, as can be seen from Fig. 1 (D-F), the register of the sarcomeres became less distinct at the height of activity. The mean sarcomere length measured during activity at L_{max} is given for the different muscles in Table 1. It can be seen that the standard deviations of sarcomere lengths measured during activity are considerably (mean 76%) higher than corresponding data obtained from the muscles at rest. This finding supports the view that there is a wider dispersion of sarcomere length during activity. The mean active sarcomere length at L_{max} determined from all 7 muscles was $2.29 \pm 0.04 \mu m$ (S.E.).

The differential behaviour of the sarcomeres along the length of the muscle was further studied by recording the distances between surface markers on the muscle. The behaviour

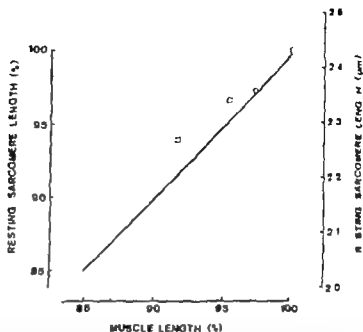


Fig. 2. Relation between resting sarcomere length (S.L.) and overall muscle length (M.L.) of 5 pp muscles. L_{max} is taken as 100 percent. The different symbols represent different muscles and each point is the mean value of 5–30 measurements. The straight line is the regression of S.L. on M.L. from the pooled data ($n=217$). Its equation is $S.L. (\mu m) = 0.0263 M.L. (\%) - 0.21$. The standard deviation about the line is $0.07 \mu m$.

of individual segments recorded in two different muscles is shown in Fig. 4. It can be seen that whereas some regions shortened, other regions actually elongated or remained constant as the muscle went from rest to full activity. An examination of different muscles failed to reveal a consistent and uniform pattern of shortening between muscles. It is noteworthy that the behaviour of the end segments was not different from that of other regions of preparation. No consistent elongation of the ends of the muscles was observed (cf. Kram and Pollack 1975). This is probably due to the fact that the metal loops used for mounting of the muscle did not come into direct contact with the muscle tissue (see Methods) to cause any damage to the contractile material of the end regions of the preparation.

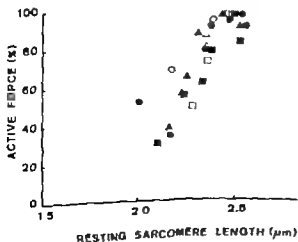
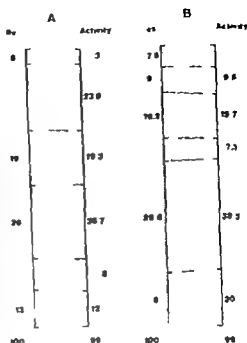
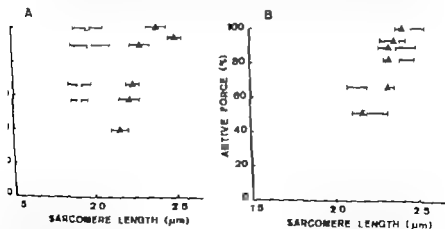


Fig. 3. Relationship between peak isometric force and resting sarcomere length in 10 pp muscles. Different symbols represent different muscles and each data point is the mean value of 5–30 measurements.

Schematic illustration of length measurements in two papillary muscles (A and B) at rest (contracted) and during activity (peak tension). Measurements from plots records of surface markers, s denote the distance between adjacent and are expressed as percent of max muscle length at L_{max} . The total length s takes as the distance between points of attachment of the rigid shanks that were used for mounting the muscle. Dashed lines indicate corresponding s at rest and peak activity. The upper part of the tracings contain the map the muscle and the tendon. Note the same behaviour of the different regions the length of the muscle as all in the two preparations.



difference in behaviour of sarcomeres during activity is further illustrated in Fig. 5 A, which presents data from two different muscles. In each muscle the mean sarcomere length in a selected region of the preparation has been determined at rest and during activity. Values at different overall lengths of the muscle are plotted against peak twitch force (Fig. 5 B). It is seen in Fig. 5 A that the sarcomeres studied in this particular case shortened by



5 Relationship between peak isometric force and resting (filled triangles) and active (open triangles) sarcomere lengths measured in one region of two different papillary muscles (A and B). Note: 1. the exponential relationship between active force and resting sarcomere length in the two preparations and 2. the tendency to sarcomere length recorded in the selected areas of the two muscles during activity.

approximately $0.35 \mu\text{m}$ as the muscle went from rest to peak activity at the various ν lengths. By contrast there was a slight elongation of the sarcomeres during activity in a muscle segment studied in the experiment of Fig. 5 B. As shown in Table 1 the rest sarcomere length during activity to that at rest varied between 0.88 and 1.01 in the 7 mm investigated.

Stretching of the tendinous attachments of the papillary muscle could generally be served during contraction. In one of the muscles the collagenous fibrils in the tendon could be clearly visualized. During rest the tendinous fibrils were arranged in a wavy pattern. With the muscle contracted, the waves straightened out. This arrangement is likely to be responsible for a substantial portion of the series compliance in the preparation used.

Discussion

Resting sarcomere lengths in the living papillary muscle preparation are quite uniform as to be expected, significantly longer than those reported for fixed material at L_{max} (Somblick *et al.* 1963; Grimm and Whitehorn 1968; Grimm *et al.* 1970).

In previous work (Grimm and Wohlfart 1974) utilizing the rat papillary muscle, a minimum sarcomere length of $2.38 \pm 0.004 \mu\text{m}$ was found, a value that is slightly though significantly less than the $2.44 \pm 0.01 \mu\text{m}$ of the present study ($p < 0.01$). Since basically the same experimental approach was used in both studies, this difference is probably real. In the experimental design of the present experiment the muscles were to be repeatedly stretched and shortened. Anticipating the possibility of some slippage (which, however, could not be statistically demonstrated) we chose as our L_{max} (see Methods) that optimum length at which the tension was greatest. As is evident from Fig. 3 the tension-length relationship exhibits a flat region between 2.4 and $2.5 \mu\text{m}$. In the previous study the minimum optimum length was chosen. We feel that the most likely explanation for this difference between the previous and present results lies in the difference between the minimum and the maximum optimum lengths.

A great part of the present experimental results depends upon the integrity and stability of our papillary muscle preparation. Since the muscle was tied via a block of ventricular muscle at one end and via the tendinous attachments at the other end, the papillary muscle itself presumably was not damaged. This assumption is supported by the normal microscopic appearance of the sarcomeres at both ends of the preparation. During surgical removal extreme care was taken not to touch the muscle with metal instruments nor to damage the muscle by overstretching nor to allow the muscle to shorten excessively. After equilibration of the muscle at the given stimulation frequency the preparation was mechanically stable for many hours.

Sarcomere shortening as expected, occurred during the mechanical twitch. The compliance of the transducer ($< 0.5\%$) resulted in only a very small part of the total. Most of the compliance existed within the preparation. One source of the compliance was directly served to be at the tendinous end and could be visualized in the microscope as a straightening of wavy collagenous fibrils. Another source of compliance probably resides in the piece of the ventricular wall used for attachment of the muscle. It should be noted that the

ting of length segments between external markers was less than the sarcomere shortening; the endocardial layer (very thin in these animals) appeared to act as a sleeve over the muscle fibres. Sources of myocardial compliance, in addition to those mentioned above, have been suggested to be the interlocking cellular arrangement, the intercalated discs and Z discs. The magnitude of any of these sources is difficult to assess at the present time. The results showed that, though resting sarcomere lengths tended to be very uniform throughout the preparation, the extent of shortening during force development was quite variable; the sarcomeres in some areas were even found to elongate. It is tempting to speculate that the intertwining nature of the myocardial cells provides for the even distribution of stresses placed upon them and that, in the relaxed state, this process is complete. During activity the extent of shortening will depend upon the compliance seen by the individual contractile units and, apparently, this compliance is quite variable within the papillary muscle. This difference in local compliance need not reflect an uneven distribution of passive elastic components within the preparation. The possibility exists that the contractile strength varies along the length of the fibre bundles in the muscle due to differences in cross-sectional area and/or regional differences in activation. This means that one region may shorten more actively at the expense of weaker segments that act in series. The increased distribution of sarcomere lengths during activity were also visualized by means of the laser diffraction technique described by Clew-orth and Edman (1972). The first-order diffraction pattern from the papillary muscle broadened and became much less distinct as the muscle went from rest to activity. Similar observations have been reported by Krueger and Pollack (1975) in studies of rat papillary muscles.

It is of interest to note that maximum force was obtained at substantially longer sarcomere lengths (mean 2.44 μm passive length and 2.29 μm active length) than during tetanic contractions in skeletal muscle. Active force was reduced to a low level (about 1/3 of the maximum) by decreasing the sarcomere length to 2.0 μm , to a length where tetanic force is maximum in frog skeletal muscle. These findings agree well with results obtained by Krueger and Pollack (1975) in studies on rat papillary muscle and by Winegrad (1974) in experiments on frog atrial preparations. However, a quantitatively different relationship between active force and sarcomere length has been reported by Julian and Sollins (1975) in rat papillary muscles; they report clearly shorter sarcomere lengths (both active and passive) at L_{max} .

The observed difference between the length-tension relationships in cardiac and skeletal muscle are not readily explainable on the basis of a difference in length of the A and I filaments between the two preparations (Page 1974). A more plausible explanation takes account of the fact that the cardiac length-tension curve refers to twitch responses rather than time-dependent tetanic contractions. Evidence from previous studies on heart muscle (Edman and Nilsson 1968, Blanks 1970, Nilsson 1972, Allen, Jewell and Murray 1974) do suggest that both the intensity and the duration of activation are increased by increasing the length of the preparation. Stretching the muscle to a longer length may increase the amount of intracellular calcium that is released in response to the action potential (cf. Edman and Kiesling 1971). On this basis then the twitch amplitude may increase with increasing sarcomere length even when exceeding the length at which the area of overlap between the A and I filaments is maximum (also see Close 1972). Conceivable mechanisms by which length may affect the

approximately $0.35 \mu\text{m}$ as the muscle went from rest to peak activity at the various lengths. By contrast there was a slight elongation of the sarcomeres during activity in the muscle segment studied in the experiment of Fig. 5B. As shown in Table 1 the ratio of sarcomere length during activity to that at rest varied between 0.88 and 1.01 in the 7 mm investigated.

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The Interrelation between Hypothalamically Induced Changes in Sympathetic Discharge to the Gastrointestinal and Cardiovascular Systems

By

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Abstract

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Unconscious-anesthetized, adrenalectomized cats, the sympathetically conveyed effects of topical hypogastric stimulations on gastric volume, blood pressure and heart rate were systematically explored. The vagus nerves were cut but could be kept active by graded afferent stimulation. In the absence of such vagal activity, hypothalamic stimulation had no appreciable influence on gastric volume, even though the stomach showed considerable myoelectric tone. When, however, vagal efferent activity was present, hypogastric stimulation could markedly affect gastric tone, indicating that the sympathetic fibres exert their major influence on the stomach via cholinergic intramural neurons. Hypothalamic stimulations that had reductions in pressure or heart rate also usually caused an enhancement of gastric tone. Secondary responses are associated with decreases in gastric volume whereas stimulation-induced tachycardia was not linked to any particular type of gastric response. Thus, the hypothalamic sympathoadhibitory actions on the cardiovascular system seem closely connected to suppression of the sympathetic outflow to the stomach. There is, on the other hand, no regular association between hypothalamic sympathoadhibitory influences on the cardiovascular and gastrointestinal systems. In fact, there is in many areas a suppression of sympathetic discharge to the stomach in association with cardiovascular stimulation.

It is well known that the sympathetic adrenergic fibres induce inhibition of gastrointestinal activity except in the sphincter regions. In most species, notably cat and man, the sympathetic fibres make their essential inhibitory connections with the cholinergic excitatory regional neurons, with only insignificant influences on gastrointestinal smooth muscle (in directly) (cf. Furness and Costa 1974). Consequently, cholinergic excitatory activity has to be present if gastrointestinal motility should be used as an indicator of changes in the activity of the regional adrenergic fibre supply. Further, the effects of vagotomy or of atropine administration have for such reasons to be interpreted with great caution in the identification of peripheral pathways mediating centrally elicited motility responses.

The stomach is a suitable organ for the study of sympathetic influences on the gastrointestinal tract. Following acute vagotomy there is hardly any activity in the cholinergic excitatory intramural neurons (Jansson and Lisander 1969). Therefore, it is experimentally possible to induce any desired level of "background" cholinergic activity in the stomach by

metabolism of activator calcium in the excitation-contraction coupling in cardiac muscle recently been discussed by Fabiato and Fabiato (1976)

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or Artificial respiration was maintained with respiration pump. — In two expts. atropine was sulphate, Merck) or guanethidine (Isorelin Ciba) as given. — In all expts. the vagi were dissected free in the neck, cut and arranged for graded stimulation in the efferent direction.

Grass S 5 stimulator to elicit increased gastric motility via the vagal excitatory fibres. The range of stimulus parameters as therefore chosen as 0.5–10 Hz, 1–2 ms and 4–15 V.

perforator stimulation. The head of the animal was placed Horsley–Clark apparatus. A monopolar stainless steel electrode was inserted into the hypothalamus. A constant current stimulator delivered regular pulses of 1 ms duration and 0.1–0.3 mA amplitude (1–4 V). The frequency was varied within limits (5–100 Hz). To identify the stimulation points, direct anodal current of 1 mA was passed through the electrode for 30 ms each track and usually at H 2. The brain was then perfused with 10% formalin.

a. From sections, 30 μ m thick, were cut and photographed under high magnification. The stimulation points were localized with reference to the end points of the tracks, the Horsley–Clarke coordinates and the locations of the identified lesions. Each stimulation point in the individual expts. was then related to the standard maps in the stereotaxic atlas of Sander and Notman (1961). Thus, the error in localization of the points may amount to 0.5 mm.

Recordings of efferent responses. Gastric volume as recorded with large rubber balloon introduced through the oesophagus (for details see Jansson 1969). The intragastric pressure as in the individual expts. was constant, at level between 8–18 cm H₂O. The system was filled with body and water and the box was connected to container placed on Statham strain gauge, recording the weight of the water into the stomach. The strain gauge was connected to Grass polygraph. In this way nonoperatively and changes of gastric volume could be continuously followed at largely constant transmural pressures. *Pressure* as recorded by Statham P 23 AC transducer connected to catheter in femoral artery and rate was recorded on Grass tachograph unit, triggered by the arterial pulse curve.

Experimental procedures. In 17 cats, the interrelationship between the changes in blood pressure, heart rate and gastric volume were studied. In one and the same animal, up to three electrode tracks were stimulated at 30 Hz, 1 ms and 1–2–0.3 mA. The three tracks were L 1, 2 and 3 in given frontal plane (A 10–14), each track the electrode was lowered to H –6 and then stimulation as performed at every 10 min during prime withdrawal of the electrode up to H –2. Each of these points was stimulated in at least 3 animals, 4 and without simultaneous vagal activation. At the end of the series, 4 cats were systematically stimulated throughout the locations A 10–14, L 1, 3, H –4 to –6 to compare in one and the same animal the system from initial parts of the hypothalamus. The changes in heart rate and blood pressure to hypothalamic stimulation without efferent vagal stimulation were compared to the changes in gastric volume during subsequent efferent vagal stimulation since this latter procedure is prerequisite to reveal adequately mediated changes in volume.

In 3 of the topical stimulations biphasic gastric responses were observed, first with both positive and negative deflections. When this was the case, the largest deflection was used for the calculations with corresponding sign. Each stimulation lasted at least 30 s but was always kept long enough to let the response develop fully. The maximum changes in stomach volume were always compared to the maximum increases, or decreases, in blood pressure and heart rate. If these two latter parameters were biphasic, each occurred only occasionally the phase was used which corresponded in time to the maximal change in stomach volume.

Statistical treatment of data. The interrelationships between the changes in adrenergic fibre discharge are examined statistically on the basis that under the present experimental conditions an increased stomach volume implied an increased sympathetic discharge, as was the case with pressure or heart rate increase. The procedure is best illustrated by an example. When it was to be determined whether, for instance, blood pressure increases were predominantly related to decreases or increases in heart rate, the number of prior responses with tachycardia, on one hand, and on the other prior responses with bradycardia were determined in each cat. These numbers were expressed in per cent of the total number of stimulations performed in the individual animals. Thus, the differences between these two percentages, with their corresponding signs, were calculated for all the animals and used in Wilcoxon test at the 0.01 and 0.05 levels.

Results

1. General character of the responses. As earlier described the vagi were cut and arranged for efferent stimulation of the gastric excitatory fibres in all expts. The hypothalamus could substantially influence gastric volume in either direction if stimulated against a background

graded efferent vagal stimulation, which is particularly advantageous in studies of the efferent reflex involvement of the regional adrenergic nerve supply.

Hypothalamic influences on gastric motility have been studied by several investigators (Thomas and Baldwin 1968) but fairly little interest has been devoted to the participation of sympathetic fibres *per se* in such gastric adjustments. One exception is the study by Lass-Lisander and Martinson (1969) in cats where the attention was focused on some functionally well defined hypothalamic regions, *i.e.* the defence area with adjacent pressor region and the nearby sympatho-inhibitory area. Stimulation of the defence area induced a marked adrenergic activation of the cardiovascular system, a cholinergically mediated vasodilation in skeletal muscle and an activation of the adrenergic fibres to the stomach, expressing itself as a volume increase. The hypothalamic pressor responses, characterized by an increased and vasoconstrictor fibre activation without any engagement of the vasodilator fibres, likewise caused an increased adrenergic influence on the stomach, resulting in a volume increase. A topical stimulation of the hypothalamic sympatho-inhibitory area, on the other hand led to a generalized inhibition of adrenergic fibre tone causing a fall in blood pressure and heart rate and a decrease in gastric volume. Thus, when these three hypothalamic areas were stimulated the adrenergic fibre activity to the gastrointestinal tract was repeatedly altered in parallel with that to the cardiovascular system. It should be stressed, however, that the sites thus studied represent only a minor fraction of the hypothalamic structures.

For such reasons the question arises whether a similar parallelism in sympathetic discharge to the cardiovascular and gastrointestinal systems is encountered also when other hypothalamic structures are stimulated. There are, indeed, reasons to believe that this may be the case mainly because there are such considerable differences at the medullary and spinal levels with respect to the organization of the cardiovascular and gastrointestinal adrenergic control. For example, the cardiovascular control is under a strong supraspinal excitatory influence implying that the activity in peripheral adrenergic 'cardiovascular' neurons is drastically diminished following spinalization (*cf.* Folkow and Neil 1971). On the other hand, the sympathetic nerves to the gastrointestinal intramural ganglia, on the other hand, form an efferent link in spinally mediated reflexes. Their reflex activity is under a supraspinal modulation whose net influence is inhibitory in nature and the reflex excitability is therefore enhanced following spinal cord transection (*cf.* Furness and Costa 1974). Such differences in the bulbospinal control of the cardiovascular and gastrointestinal adrenergic supply may be related to the possibility that their control may in important respects be differentiated also at the hypothalamic level. In the present paper a more systematic exploration was therefore performed with respect to the hypothalamic influence on the adrenergic fibre discharge to the cardiovascular system and to the stomach, here representing the gastrointestinal system.

Methods

The experiments were performed on 30 cats of either sex, weighing between 2.1 and 3.5 kg. The animals were deprived of food for 24 h before the experiments. After induction with ether they were anaesthetized with chloralose, 50 mg/kg b.wt. A tracheal cannula was inserted. After midline laparotomy the adrenals were approached transabdominally and ligated to eliminate the release of catecholamines into the blood stream. Adrenocortical substitution was given by i.v. injection of hydrocortisone (Solu-Gluc, Ercos, 10 mg/kg). Gallamine triethiodide (Flaxedil, May and Baker 4 mg/kg i.v.) was used to relax the somatomotor system.



FIG. 2. 6 kg Vag. cut and arranged for afferent stimulation (3 Hz, 1 sec, 4 V). Hypothalamic ethanols-A13, L3, H 4 and 5. Note that the afferent vagal stimulation evokes excitatory gastric responses, cholinergic stimulation (30 Hz, 1 sec, 0.3 mA). Thus, gastric excitation is here elicited together with a blood pressure and heart rate.

blood pressure were more often associated with increases in heart rate ($p < 0.01$) than any single type of heart rate response and vice versa.

Adrenergically mediated changes in gastric volume were however not so well correlated to the changes in pressure and heart rate as these latter changes were mutually correlated. Only when depressor or bradycardia responses were induced, these cardiovascular responses were most frequently associated to decreases in gastric volume ($p < 0.01$), reflecting a generalized inhibition of adrenergic fibre activity to both the cardiovascular and gastrointestinal systems. The hypothalamically induced reductions in blood pressure, heart rate and in gastric volume were without exception slow in onset but could, in a period for several minutes after cessation of stimulation. Thus, the suppressed sympathetic activity regained its tonic activity first after such a long latency that a reverberating inhibitory influence might be suspected.

* Attention was a different one concerning the hypothalamic points from which pressor tachycardia responses were elicited. Thus, blood pressure increases were linked to rises in stomach volume ($p < 0.01$), indicating a differentiated sympathetic influence on cardiovascular and the gastrointestinal systems. Concerning the points eliciting heart increases, these responses were not significantly connected with any particular type of gastric response ($p > 0.2$). However in the majority of these hypothalamic points the direction of changes in sympathetic discharge to the cardiovascular and gastrointestinal systems opposite to each other which, as mentioned, was not the case when the sympathetic output to the cardiovascular system was depressed.

In the data, stimulations in the ventral hypothalamus were overrepresented (see Methods). Further analysis, however revealed interrelations between the variables of essentially the same character in these ventral parts as in the more dorsal regions.

Localisation of response sites. Each hypothalamic point in this systematic study was stimulated in 3-6 cats and the responses were averaged. A topographical

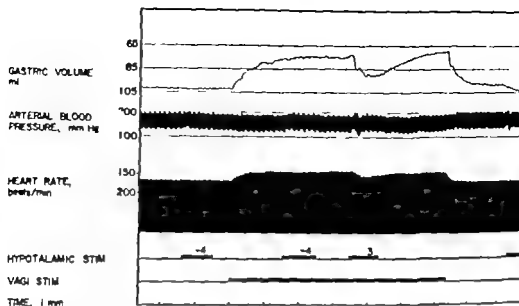


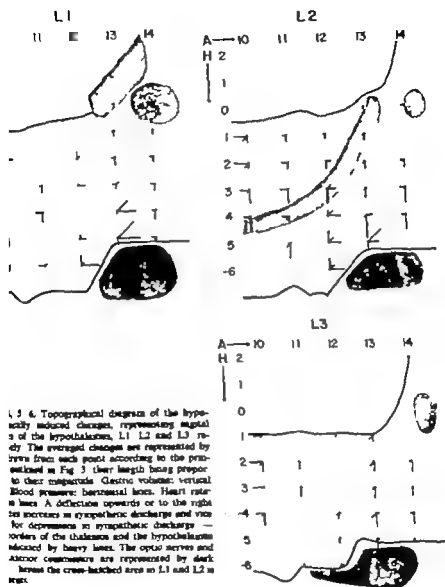
Fig. 1 Cat 2.2 kg. Vagi cut and arranged for efferent stimulation. Hypothalamic stimulation at # 1 ms and 0.2 mA. 1 A10, L1 H -4 and -3. Note the different character of the responses here the electric stimulus is modified one mm and also that efferent vagal stimulation (2 Hz, 1 ms, 8 V) is a prerequisite to demonstration of the strong gastric inhibition at -3.

of a prevailing efferent vagal stimulation but was virtually ineffective if activated a (see Fig. 1 and 2). The frequency-effector response relationships of these hypothalamic stimulations during efferent vagal stimulation at 1-4 Hz were studied in 5 cats. Increases in stomach volume, reflecting an increased sympathetic discharge, were maximal at 60-80 Hz (3 points, 2 cats) whereas decreases in volume, due to inhibition of sympathetic activity, were maximal already at 40 Hz (5 points, 3 cats).

Pharmacological blockade of the gastric responses was performed in two cats. In one animal the effect of guanethidine (4 mg/kg) was tested on the decreases in gastric volume during hypothalamic stimulation which were blocked by this drug. In the other animal atropine (1 mg/kg) was used which also blocked the decreases in gastric volume thus confirming observations and analysis earlier performed by Jansson, Lisander and Martinson (1969).

2 Interrelationships between cardiovascular and gastric changes. The hypothalamus was systematically explored by topical stimulations in 17 cats. Altogether 464 points were stimulated and the sympathetically mediated changes in gastric volume, blood pressure, heart rate were recorded. The interrelation between the changes observed are given in Table 1. For each experiment, the changes in the variables recorded at each stimulation were plotted as follows: Blood pressure versus heart rate, blood pressure versus stomach volume, heart rate versus stomach volume. The data were then categorized according to the constellation of changes and the number in each group in the table was expressed in relation to the total number of stimulations in each cat on a percentual basis. In the Table are given averaged percentages in the various groups for all the 17 cats, together with the standard deviation.

The changes in heart rate were well correlated to those in blood pressure. Thus



ular function, in order to find out to what an extent they were linked together in a far fashion when the various hypothalamic sites were excited. The mean values presented in Fig. 4 therefore reflect both the direction and the average magnitude of the individual, pathetically conveyed responses within the cardiovascular and gastrointestinal systems. They agree well in their general character with the stimulation responses in individual cases, with only few exceptions. They also confirm the Table insofar as depressor-cardiac responses were regularly linked to decreases in gastric volume as were pressor

TABLE I The interrelationships between the cardiovascular and gastric changes, on a per cent BP, HR and SV denote blood pressure, heart rate and stomach volume, respectively. The sign of a variable is indicated by the sign = 0, an increase by > 0 and a decrease by < 0. For information see text.

BP-HR								
BP=0	BP>0	BP<0	BP=0	BP=0	BP=0	BP>0	BP=0	BP=0
HR=0	HR=0	HR=0	HR>0	HR<0	HR>0	HR<0	HR=0	HR=0
40.29	10.4	10.29	0.49	1.24	15.91	3.25	16.36	1.07
±18.04	±11.15	±6.66	±2.01	±3.81	±16.2	±8.20	±13.80	±3.87
BP-SV								
BP=0	BP>0	BP<0	BP=0	BP=0	BP>0	BP>0	BP=0	BP=0
SV=0	SV=0	SV=0	SV=0	SV<0	SV>0	SV<0	SV=0	SV=0
34.47	7.06	6.56	1.14	7.04	4.59	17.77	17.71	3.61
±14.68	±10.22	±7.61	±4.68	±7.05	±8.66	±17.71	±16.90	±3.14
HR-SV								
HR=0	HR>0	HR<0	HR=0	HR=0	HR>0	HR>0	HR=0	HR=0
SV=0	SV=0	SV=0	SV>0	SV<0	SV>0	SV<0	SV<0	SV=0
42.09	2.19	3.79	2.01	17.33	5.41	10.63	14.53	1.94
±13.53	±4.20	±6.04	±3.94	±11.90	±9.15	±12.97	±12.69	±4.06

representation of the effects of these stimulations is presented according to the plan outlined in Fig. 3. Thus, *increases* in sympathetic discharge are indicated as 1) upward vertical deflections for gastric volume increases, as 2) upward oblique deflections for heart rate increases and as 3) horizontal deflections to the right for blood pressure increases. *Decreases* in sympathetic activity the respective lines go in the opposite direction. The length indicate the relative magnitude of the neurogenic effector responses. These plots are used for the construction of the response maps in Fig. 4 indicating the patterns of responses obtained from the various hypothalamic sites at 1 mm, 2 mm and 3 mm lateral to the midline. These maps illustrate the main aim of the study *i.e.* the exploration of relationships between the sympathetically mediated changes in gastric volume and in

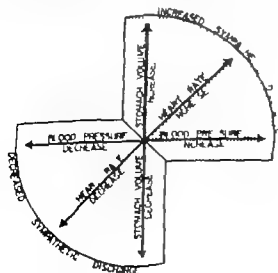


Fig. 3 The principal arrangement of the response maps used for the effector responses in the response point in Fig. 4. For further explanations see text.

in the absence of any prevailing vagal activity. Moreover, Jansson, Linder and Marz (1969) found that the alpha-adrenergic blocking drug, phenoxybenzamine, markedly altered the cardiovascular adrenergic responses to hypothalamic defence area and pressor stimulation but did not alter the gastric motility responses.

In the present experiments, the carotid baroreceptors were left intact and their activity may have been changed by the cardiovascular adjustments. However, these receptors do not have any direct reflex effects on the adrenergic outflow to the smooth muscle of the stomach wall (Jansson and Martinson 1966, Abrahamsson and Thorén 1972). Thus, it appears unlikely that such reflexes should have contributed to the hypothalamically induced gastric responses. In 11% of the stimulations, there were cardiovascular changes without any consistent alteration of gastric tone. This may simply mean that most hypothalamic structures influence the sympathetic nervous system produce changes both in cardiovascular and gastrointestinal functions. It is further obvious that topical stimulation may excite neurons and pathways which are not necessarily normally involved in joint actions and such an error increases with rising stimulation intensity.

It is, however, of greater interest to consider also the direction of the changes. With respect to depressor and bradycardia responses, indicating a reduction of sympathetic discharge to the cardiovascular system, this reduced discharge showed a close correlation to a reduced sympathetic discharge also to the gastrointestinal tract ($p < 0.01$). The great majority of blood pressure reductions noted in the present study were slow in onset, lasted for the duration of the stimulation, had a long poststimulatory recovery period and were regularly associated with reductions in heart rate. This is most characteristic of the sympatho-inhibitory reaction (Folkow, Johansson and Öberg 1959), where the efferent mechanism is an overall inhibition of sympathetic discharge to both heart and vessels. It is interesting to note that these obviously sympathoinhibitory responses were so closely associated with signs of inhibited sympathetic discharge also to the gastrointestinal system that it appears as if these inhibitory components are normally closely coupled at the hypothalamic level.

The functional significance of such sympatho-inhibitory responses remains obscure. Löfving found that similar cardiovascular adjustments can be elicited from the anterior hypothalamic nucleus and that the hypothalamic sympatho-inhibitory area seems to serve as a relay station for these effects (Löfving 1961). Their resemblance to the circulatory changes induced by carotid baroreceptor activation was noted and it was further suggested that the hypothalamic inhibitory area may form one of the links in a system mediating emotionally coloured responses such as fainting and the "playing dead" reaction met with in many species. Hilton and Snyder (1971) showed that destruction of the mentioned hypothalamic area caused some impairment of the baroreceptor reflexes. Therefore, they envisaged the relay station for the baroreceptor reflexes as an elongated neuron pool, stretching from the lower medulla oblongata to include also these rostral hypothalamic structures. Even if this may be the case, it by no means denies that the hypothalamic sympathoinhibitory area may well subserve also other functions, such as "emotionally charged" response patterns. Furthermore, the baroreceptor reflexes are normally devoid of any direct neurogenic influences on gastric motility (Jansson and Martinson 1966, Abrahamsson and Thorén 1972), while the hypothalamic sympathoinhibitory area has, apart from in the present study been found to

responses, whereas tachycardia responses were not systematically associated with a particular type of gastric volume change.

Points yielding cardiovascular changes only were not included in Fig. 4. They are only 11% of the whole material and the corresponding changes in heart rate and pressure were without exception minor. The points were irregularly scattered with a tendency of being located to the outskirts of the autonomically active areas.

Discussion

The main aim of the present study was to examine the degree of parallelism in the changes of the adrenergic fibre supply to the gastrointestinal system and to the cardiovascular system in response to hypothalamic stimulation. Heart rate and arterial blood pressure were used as cardiovascular variables. As the vagi were cut and the adrenals eliminated, changes in heart rate could only be explained by variations in adrenergic neurogenic tone. Increases in sympathetic discharge almost invariably cause increases of mean arterial pressure in cats, even in situations where the sympathetic cholinergic vasodilator fibres participate in the overall response as in the defence reaction (cf. Lisander 1970). Thus, in all situations increases in pressure and heart rate indicate an augmented sympathetic discharge and *vice versa*.

Under the present experimental conditions, the hypothalamus could only influence gastric volume *via* the sympathetic fibres and only if the vagi were simultaneously stimulated in the efferent direction. In confirmation of earlier findings by Jansson, Lisander and Mår (1969). They also observed that these neurogenic changes in gastric volume, as well as inhibitory, were abolished by atropine or guanethidine, as confirmed in experiments in the present series. All these findings are compatible with an intramural, paracrine point of action of the adrenergic fibres, with an inhibitory influence on the gastric cholinergic neurons rather than on the smooth muscle effectors (cf. Furness and Costa 1974).

The hypothalamically induced gastric responses were regularly associated with changes in blood pressure and heart rate. The question arises whether the changes in gastric volume might be secondary to changes in gastric circulation. There is, however, no clear relation between gastrointestinal blood flow and motility (cf. Furness and Costa 1974). Known, for example, that blood flow to the intestine had to be drastically reduced before a significant inhibition of motility occurred. Carotid occlusion caused marked pressor responses but did not change motility in decentralized intestinal loops in cats with denervated adrenals (Kock 1959). Thus, it appears less likely that changes in arterial pressure *per se* have caused any substantial gastrointestinal motility effects in the present study. This is particularly unlikely in view of the marked autoregulation of flow in the intestinal circuit, tending to keep regional blood flow constant (cf. Folkow and Neil 1971). A further strong argument is that the diencephalic stimulations affected gastric volume when a prevailing activity in the vagal excitatory fibres was maintained, despite the fact that the gastric smooth muscle possesses a considerable myogenic tone even without such vagal stimulation. In case neurogenic alterations in gastric blood flow affected the tone of the gastric smooth muscle, one would have expected such indirect changes in gastric volume.

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cause sympathetically mediated decreases in gastric volume (Jansson, Lisander and Nilsson 1969) and enhancements of colonic motility (Rostad 1973).

The question arises as to the mechanisms mediating the gastric effects from hind-area stimulation. The present expts. involve an abdominal trauma which is known to act via spinal reflexes, collectively called the gastrointestino-gastrointestinal reflex, which is sympathetically conveyed and under the influence from higher CNS levels. Thus, it has been found that these reflexes can be completely inhibited from supraspinal levels of the nervous system (Johansson, Jonsson and Ljung 1965; Martner 1975). It is therefore tempting to assume that also the hypothalamic inhibitory area can exert such an influence, but this problem requires further study.

Pressor and tachycardia responses were obtained from extensive parts of the hypothalamus and they were mutually closely correlated ($p < 0.01$). However, pressor responses commonly occurring together with a decrease in gastric volume ($p < 0.01$). The same was the case with tachycardia responses but here the association was not statistically significant ($p > 0.2$). These results strongly indicate that the hypothalamic sympathetic control mechanisms of the gastrointestinal and cardiovascular systems are sometimes changed in parallel but quite often also in opposite directions. In other words, the hypothalamic neurons seem to be organized in patterns that allow various types of combinations of efferent discharges, enabling the higher CNS levels to produce a variety of differentiated discharge patterns.

Most of the presently recorded pressor responses were encountered in medial and ventral parts of the hypothalamus, where according to Hess and Brügger (1943) aggressive behaviour is commonly elicited. The cholinergic sympathetic dilator fibres to skeletal muscle are excited from an overlapping zone, the defence area. Abraham, Hilton and Zbrozyna (1965) induced patterns of flight or attack by stimulation of this area. There were some differences with respect to the gastric responses associated with the presently observed responses. Thus, in the anterior part of the pressor zone, gastric volume was usually decreased, suggesting a reduced sympathetic discharge to the gastrointestinal tract. More posteriorly the pressor responses were associated with gastric volume increases, i.e. here there were signs of an increased sympathetic discharge to both the cardiovascular and gastrointestinal systems. Again it may be so that, dependent on the nature of the environmental stimulus and the associated emotional balance, various patterns of discharge may be elicited, sometimes exciting, sometimes depressing gastrointestinal function in association with cardiovascular excitation in situations of fear, rage etc.

The present expts. have so far only aimed at examining the sympathetic effects on blood vessels and heart. The vagal outflow to both heart and stomach may also be influenced by the hypothalamus (cf. Lisander 1975), adding to the complexity of the system. It is further to be reemphasized that in the present expts. there has in all likelihood been a high degree of background reflex activity in the sympathetic nerves to the gastrointestinal tract. Expts. on non-laparotomized cats may yield strikingly different results. It is also intended that the present results will be completed with studies on unanesthetized spontaneously behaving animals, particularly to explore in which situations a gastrointestinal excitation is associated with excitation of the cardiovascular system.

This study was supported by grants from the Swedish Medical Research Council (14X-4249) and one due to Mrs Kerstin Bongsell for excellent technical assistance.

in an earlier report, Klevmark (1974) described a new cystometric method which was used to investigate the natural filling of the intact bladder. 5 different rate-models were used to study bladder behaviour during filling at physiological rates. At low filling the pressure curve was always horizontal. At higher rates of filling a rise occurred in intravesical pressure, and the new pressure level was retained when the filling rate was fully reduced (pressure accumulation phenomenon). Results obtained using this method of cystometry were similar to those recorded during natural filling through spontaneous and stimulated urinary excretion.

The purpose of the present work was therefore, to study the effects of extrinsic bladder irritation on intramural tension (tonus) during controlled slow filling (Controlled Slow Cystometry) and on intravesical pressure patterns during natural filling. The results show that the intravesical pressure reactions present in the intact cat are also found in the excised organ.

Methods

Animals and experimental procedures. The results are based on data obtained from acute experiments on 35 cats of both sexes, weighing 2.3-4.3 kg.

The animals were anaesthetized with pentobarbital sodium (Nembutal, Abbott) in doses of 30 mg/kg. Small supplementary doses were given as required. The animals were kept in the supine position on a heated pad. The rectal temperature was maintained at about 37°C. The blood pressure was recorded with Statham pressure transducers by means of catheters inserted into the femoral artery. Thoracic vesicular secretions were recorded on the polygraph by Grass volumetric pressure transducer type 5A. The animals are breathing spontaneously through tracheostoma.

Before non-specific reactions due to surgical manipulations, exposure of the bladder to air and time to the initially injected volume, 30-40 min were allowed for adaptation, before recordings were started.

Neuraxonal procedures. Through a low midline incision, the perineuritic pelvic nerves lateral to the ureters were reached and sectioned distal to their ramification. To ensure radical denervation the closely related middle rectal artery and surrounding connective tissue were cut as well. Sympathectomy was formed by section of the hypogastric nerves and removal of the inferior mesenteric ganglion. In carrying out several hours the denervation procedures had to be done with special precautions. Denervation performed close to the bladder caused early bladder edema because of certain number of vessels had to be cut, changing bladder wall constantly and thus probably the resistance to stretch (compliance). Operations performed too caudally on the pelvic or sacral nerves involved more extensive surgery which after a few hours might result in a drop in blood pressure and in urinary excretion.

Filling procedures. Bladder filling was obtained through: (i) constant-volume infusion pump, or through (ii) urinary excretion.

Controlled Slow Cystometry (CSC) was used as described (Klevmark 1974). In short: A double catheter was inserted into the vertex of the bladder through a low midline incision. Intravesical pressure was recorded through Statham pressure transducer connected to Grass Model 7 Polygraph and related to pressure changes of 0.5 cm H₂O. A Harvard constant-volume infusion pump was used for filling the bladder with saline at 37°C. The rate used was the calculated hour-duration (HD) of intact animals using 26 ml/kg/24 h as normal value ($HD = 11 \text{ ml/kg/h}$) (Spector 1956, p. 341). Thus, normal rate of urinary excretion is the average rate during 24 h. The filling rates were in the physiological range of up to 15 times the normal rate (1-15 HD).

After perineuritic denervation did not occur and in the experiments with spontaneous excretion of urine, the average observation time was chosen as for the intact cat 7.5 h (Klevmark 1974).

Factors determining the intravesical pressure. The recorded intravesical pressure includes 3 factors (Klevmark and Clow 1974): (i) extravesical pressure (weight resting on bladder), (ii) intravesical hydrostatic pressure (weight of fluid above the catheter orifice) and (iii) pressure due to all tension. The latter pressure is only one which varies with the rate of filling during these experiments. Extravesical pressure is constant when the animal resides in the same position and has free respiration (van Gierst 1957; Klevmark 1974).

Motility of the Urinary Bladder in Cats during Filling at Physiological Rates

II: Effects of Extrinsic Bladder Denervation on Intramural Tension and on Intravesical Pressure Patterns

By

BJORN KLEVMARK

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Abstract

KLEVMARK B. *Motility of the urinary bladder in cats during filling at physiological rates. Effects of extrinsic bladder denervation on intramural tension and on intravesical pressure patterns* Acta physiol. scand. 1977 101 176-184

The influence of extrinsic innervation on bladder wall tension (tonus) and on intravesical pressure was studied with a new cystometric procedure (Controlled Slow Cystometry CSC) and during filling. After parasympathectomy but not after sympathectomy the basal intravesical pressure markedly increased. At low filling rate the partial or completely denervated bladder was filled from initial volumes without any increase in intravesical pressure. At higher rates of filling a pressure occurred, and this reaction was not affected by either parasympathectomy or sympathectomy. At least one cat the new pressure level was retained when the filling rate was gradually reduced. The intravesical pressure patterns observed during filling were preserved in the partial or completely denervated organ. Thus, bladder adaptation during natural filling occurs independently of extrinsic innervation.

Studies on regulation of intramural tension (tonus) during the collecting phase resulted in two opposite theories: the myogenic and the neurogenic. According to the myogenic theory tonus reflects the physical properties of the bladder wall and is independent of neural activity (Nesbit *et al.* 1947, Tang and Ruch 1955, Lock and Fox 1963, Sabetian 1965 b a.o.). The neurogenic theory refers to the accommodation of the bladder during filling as obtained by nervous inhibition, parasympathetic (Sherk 1915, Langworthy and Kolb 1933, Kuru and Iwanaga 1966 a o.) or sympathetic (Edvin 1968 a, Sundin and Carlsson 1972, De Groat 1975 a.o.). However, in these reports authors have been studying inhibition of the rise in intravesical pressure which is observed during filling at high and non-physiological rates. The previous experimental methods do not necessarily illustrate bladder behaviour during physiological rates of filling.

In an earlier report, Klevmark (1974) described a new cystometric method which was used to investigate the natural filling of the intact bladder. 5 different rate-models were tested to study bladder behaviour during filling at physiological rates. At low filling rates the pressure curve was always horizontal. At higher rates of filling a rise occurred in intravesical pressure, and the new pressure level was retained when the filling rate was slowly reduced (pressure accumulation phenomenon). Results obtained using this type of cystometry were similar to those recorded during natural filling through spontaneous and stimulated urinary excretion.

The purpose of the present work was therefore, to study the effects of extrinsic bladder innervation on intramural tension (tonus) during controlled slow filling (Controlled Slow Cystometry) and on intravesical pressure patterns during natural filling. The results show that the intravesical pressure reactions present in the intact cats are also found in the denervated organ.

Methods

Animals and experimental procedures: The results are based on data obtained from acute experiments on 15 cats of both sexes, weighing 2.3-4.3 kg.

The animals were anaesthetized with pentobarbital sodium (Nembutal, Abbott) *p. m.* in doses of 30 mg/kg. Small supplementary doses were given as required. The animals were kept in the supine position on a heated pad. The rectal temperature was maintained at about 37°C. The blood pressure was recorded with Statham pressure transducer by means of catheters inserted into the femoral artery. Therefore urinary secretions are recorded on the polygraph by Grass volumetric pressure transducer type 15A. The animals were breathing spontaneously through tracheostomies.

To reduce non-specific reactions due to surgical manipulations, exposure of the bladder to air and compression to the initially injected volume, 30-40 min were allowed for adaptation, before recordings are started.

Denervation procedures: Through a low median incision, the parasympathetic pelvic nerves lateral to the aorta were reached and sectioned caudal to their innervation. To ensure radical denervation the closely related middle rectal artery and surrounding connective tissue were cut as well. Sympathectomy was performed by section of the hypogastric nerves and removal of the inferior mesenteric ganglion. In eight of the several hours the denervation procedure had to be done with special precautions. Denervation performed close to the bladder caused early bladder claps because carbon dioxide of veins had to be released, changing bladder wall consistency and thus probably the resistance to stretch (compliance). Operation performed too caudally on the pelvic or rectal nerves involved more extensive surgery which after a few hours might result in a drop in blood pressure and in urinary excretion.

Filling procedures: Bladder filling was obtained through: (i) constant-volume infusion pump, or through (ii) urinary excretion.

Controlled Slow Cystometry (CSC) was used as described (Klevmark 1974). In short: A double-lumen catheter was inserted into the vertex of the bladder through a lower median incision. Intravesical pressure is recorded through a Statham pressure transducer connected to a Grass Model 7 Polygraph and calibrated to pressure changes of 0.5 cm H₂O. A Harvard multipaced constant-volume infusion pump was used for filling the bladder with saline at 37°C. The *flow-rate* was the calculated hour-diameter (HD) of the cat animals using 26 ml/kg/24 h as normal value (1 HD = 1.1 ml/kg/h) (Spector 1956, p. 341). Then, the rate of urinary excretion is the average rate during 4 h. The filling rates were in the physiological range of up to 15 times the normal rate (1-15 HD).

After parasympathectomy excitation did not occur and in the experiments with spontaneous excretion of urine, the average observation time was chosen as for the intact cat 7.5 min (Klevmark 1974).

Factors determining the intramural pressure: The recorded intravesical pressures includes 3 factors (Klevmark and Clow 1974): (i) extravesical pressure (weight resting on bladder), (ii) intravesical hydrostatic pressure (weight of fluid above the catheter orifice) and (iii) pressure due to all tensions. The latter pressure is the only one which varies with the rate of filling during these experiments. Extravesical pressure is constant when the animal resides in the same position and has free respiration (see Garavito 1957, Klevmark 1974).

Motility of the Urinary Bladder in Cats during Filling at Physiological Rates

II Effects of Extrinsic Bladder Denervation on Intramural Tension and on Intravesical Pressure Patterns

By

BJORN KLEVMARK

Received 23 March 1977

Abstract

KLEVMARK B *Motility of the urinary bladder in cats during filling at physiological rates. Effects of extrinsic bladder denervation on intramural tension and on intravesical pressure patterns* Acta physiol. scand. 1977 101 176-184

The influence of extrinsic innervation on bladder wall tension (tonus) and on intravesical pressure was studied with a new cystometric procedure (Controlled Slow Cystometry CSC) and during filling. After parasympathectomy but not after sympathectomy the basal intravesical pressure markedly increased. At low filling rate the partially completely denervated bladder was filled to initial volumes without any increase in intravesical pressure. At higher rates of filling a pressor occurred, and this reaction was not affected by either parasympathectomy or sympathectomy. In intact cat, the new pressure level was retained when the filling rate was gradually reduced. The intravesical pressure patterns observed during filling were preserved in the partially or completely denervated organ. Thus, bladder adaptation during natural filling occurs independently of extrinsic innervation.

Studies on regulation of intramural tension (tonus) during the collecting phase resulted in two opposite theories: the myogenic and the neurogenic. According to the myogenic theory tonus reflects the physical properties of the bladder wall and is independent on neural activity (Nesbitt *et al.* 1947, Tang and Ruch 1955, Kock and Fox 1963, Sabetian 1965 b a.o.). The neurogenic theory refers to the accommodation of the bladder during filling as obtained by nervous inhibition, parasympathetic (Sherrill 1915, Langworthy and Kolb 1933, Kuru and Iwanaga 1966 a.o.) or sympathetic (Edwards 1968 a, Sundin and Carlsson 1972, De Groat 1975 a.o.). However, in these reports authors have been studying inhibition of the rise in intravesical pressure which is obtained during filling at high and non physiological rates. The previous experimental methods do not necessarily illustrate bladder behaviour during physiological rates of filling.

In an earlier report, Klevmark (1974) described a new cystometric method which was used to investigate the natural filling of the intact bladder. 5 different rate-models were used to study bladder behaviour during filling at physiological rates. At low filling the pressure curve was always horizontal. At higher rates of filling a rise occurred in intravesical pressure, and the new pressure level was retained when the filling rate was usually reduced (pressure accumulation phenomenon). Results obtained using this type of cystometry were similar to those recorded during natural filling through spontaneous and stimulated urinary excretion.

The purpose of the present work was therefore, to study the effects of extrinsic bladder innervation on intramural tension (tonus) during controlled slow filling (Controlled Slow Cystometry) and on intravesical pressure patterns during natural filling. The results show the intravesical pressure reactions present in the intact cats are also found in the isolated organ.

Methods

Animals and operational procedures. The results are based on data obtained from acute experiments on 35 cats of both sexes, weighing 2.3-4.3 kg. All animals were anaesthetized with pentobarbital sodium (Membrolan, Abbott) in doses of 30 mg/kg. Small supplementary doses were given as required. The animals were kept in the supine position on a heated pad. The rectal temperature was maintained at about 37°C. The blood pressure was recorded with Statham pressure transducer by means of catheters inserted into the femoral artery. Thoracic respiratory movements were recorded on the polygraph by Grass volumetric pressure transducer type 1A. The animals were breathing spontaneously through tracheostomies.

Before any specific reactions due to surgical manipulations, exposure of the bladder to air and to the usually injected volumes, 30-40 min were allowed for adaptation, before recordings were made.

Innervation procedures. Through low midline incision, the parasympathetic pelvic nerves lateral to the ureters are reached and sectioned distal to their innervation. To ensure radical deservation the closely associated middle rectal artery and surrounding connective tissues were cut as well. Sympathectomy was carried out by section of the hypogastric nerves and removal of the inferior mesenteric ganglion. In experiments involving lower dissections procedures had to be done with special precautions. Deservations performed close to the bladder caused easily bladder edema because certain number of veins had to be ligated, changing bladder wall consistency and thus probably the resistance to stretch (compliance). Operations performed too centrally on the pelvic or sacral nerves involved more extensive surgery which after 10 hours might result in drop in blood pressure and in urinary excretion.

Filling procedures. Bladder filling was obtained through (i) constant-volume infusion pump or through (ii) urinary excretion.

Controlled Slow Cystometry (CSC) was used as described (Klevmark 1974). In short. A double-lumen catheter was inserted into the vertex of the bladder through low midline incision. Intravesical pressure was recorded through Statham pressure transducer connected to Grass Model 7 Polygraph and recorded to pressure changes of 0.5 cm H₂O. A Harvard multistage constant-volume infusion pump was used for filling the bladder with saline at 37°C. The rate used was the calculated hour-diameter (HD) of intact animals being 26 ml/kg/4 h as normal value (1 HD = 11 ml/kg/h) (Spector 1956, p. 341). Then, final rate of urinary excretion, the average rate during 24 h. The filling rates are in the physiological range of up to 15 times the normal rate (1.15 HD).

After parasympathectomy micturition did not occur and in the experiments with spontaneous excretion of urine, the average observation time was chosen as for the intact cat 7.5 h (Klevmark 1974).

Factors determining the intramural pressure. The recorded intravesical pressure includes 3 factors (Terrell and Clew 1974). (i) extravesical pressure (weight resting on bladder), (ii) intravesical hydrostatic pressure (weight of fluid above the catheter orifice) and (iii) pressure due to wall tension. The latter pressure is the only one which varies with the rate of filling during these experiments. Extravesical pressure is constant for the animal voids in the same position and has free respiration (von Garrait 1957, Klevmark 1974).

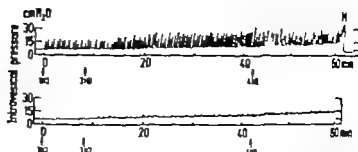


Fig. 1 Cat 2.8 kg. Intravesical pressure before (top) and after sympathectomy (bottom) in response to controlled slow filling. Initial bladder volume 5 ml. Filling to the same final volume with increasing rates as indicated (1.3 and 4 HD). No rise in intravesical pressure was recorded at normal rate of fill (1 HD), but a gradual increase occurred at higher rates (3-4 HD). No change in intravesical pressure before and after sympathectomy. Volume threshold for micturition (M) was not lowered. Note the rise in spontaneous rhythmic activity following denervation (see text). The rate-unit (HD) = 1 ml/kg (Methods).

Recording of the hydrostatic factor is reduced by short insertion and fixation of the catheter through top of the bladder. Increase in hydrostatic pressure is kept constant during the experiments in the same cat filling to the same final volume. Thus, when the length (volume) factor of intramural tension (Laplace's equation (Klevmark 1974)) is kept constant, a change in intravesical pressure represents a change in intramural tension.

Comment on the concept of tonus

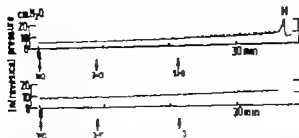
Intramural tension and tonus are identical concepts. However tonus is used more generally. Intramural tension is usually restricted to walls of hollow organs. Difficulties exist in operationally defining tonus in mechanical terms. Theoretically comparison of pressure-volume or tension-length measurements should indicate the degree of tonus reliably provided that the rate of distension is as close to the physiological range of the actual organ (Alexander 1957). Bladder tonus has been operationally defined as resistance to stretch (Evans 1936) or as the slope of the pressure-volume curve obtained by cystometry (Taig and Ruch 1953). As already mentioned, the rate of distension has usually been outside the physiological range of the bladder. Recently the term compliance has been proposed as a substitute for bladder tonus, indicating the change in volume for a change in pressure (Held et al 1971).

Results

Experiments described under (A) were performed to study bladder tonus in response to controlled slow filling before and after partial or complete denervation. The bladder was filled from an initial volume of 5 ml to the same final volume before and after operation in the same cat. Filling was performed with infusion pump at rates between 1-15 HD. Tubings inserted through the bladder top (see Methods). In (B) the intravesical pressure patterns demonstrated in intact cats were investigated after complete extrinsic denervation using natural filling through spontaneous and stimulated urinary excretion. The bladder was filled from an initial volume of 5-10 ml. Experiments in (C) were of methodological nature.

A Effects of extrinsic bladder denervation on intramural tension (tonus) in response to controlled slow filling

(1) Sympathectomy: Fig. 1 shows the intravesical pressure reactions in a typical cat before and after denervation during increasing rates of filling. In all 7 cats no rise in pressure



2 Cat 3.2 kg. Intravesical pressure before (top) and after parasympathectomy (bottom) in response to controlled slow filling. Initial volume 5 ml and filling to equal final volume with increasing rates (1.3 and 11 HD). No rise in intravesical pressure at normal rate of filling (1 HD), but gradual increase occurred at higher rates (3-4 HD). After denervation the basal pressure was markedly elevated, but the rise in pressure at higher rates was unchanged. Micturition (M) was abolished.

observed at normal filling rate (1 HD). At higher rates of filling (3-4 HD in Fig. 1) the intravesical pressure gradually increased. However the intravesical pressure changes to the extent rates of filling were identical in the intact and sympathectomized bladder. Volume threshold for micturition was not lowered. Spontaneous rhythmic contractions either decreased or became irregular with low amplitude, and showed no significant response to such. The same changes of spontaneous rhythmic activity occurred after parasympathectomy and after complete denervation.

2) *Parasympathectomy*. At normal rate of filling (1 HD) no rise in the intravesical pressure was observed in the intact or denervated bladder (Fig. 2). The pressure increases at higher rates of filling (3-4 HD in Fig. 2) were also identical in the two situations. In all 4 cats the basal pressure increased 2-4 cm H₂O after denervation. Micturition was abolished.

3) *Complete denervation*. In all 3 cats which were parasympathectomized and sympathectomized no rise in basal pressure was observed (Fig. 3). No rise in intravesical pressure was observed at normal rate of filling (1 HD). At higher rates the pressure increase was more even as shown in Fig. 3. For the total observation time no overall difference in the pressure reactions was observed in the intact and completely denervated bladders. Micturition was abolished.



3 Cat 3.4 kg. Intravesical pressure before (top) and after complete extrinsic denervation (bottom) in response to controlled slow filling. The initial volume was 5 ml and filling up to equal final volume as performed with increasing rates (1.3 and 11 HD). No rise in pressure as observed at normal rate of filling (1 HD). For the total observation time (0-45 min) no difference in total pressure rise was observed. Micturition (M) was abolished.

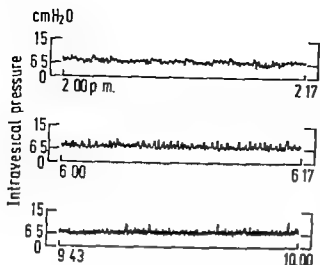


Fig. 4 Cat 2.1 kg. Intravesical pressure & natural filling at low rates (spontaneous urinary excretion) after complete extrinsic denervation. The initial bladder volume 10 ml and the tracings show 3 separate periods. No increase in intravesical pressure during filling for 8 h with 26 ml of urine.

II Effects of complete extrinsic denervation on intravesical pressure patterns during natural filling

(1) *Filling by spontaneous urinary excretion* The intravesical pressure was recorded in 5 cats with an initial volume of 10 ml of saline. In 5 cats the arterial blood pressure (abv 120 mmHg) and urinary excretion remained satisfactory during the experimental period (2½ h to 9 h, average 7½ h). Average urinary excretion varied from 1–2 HD in 4 cases and was ½ HD in one. No significant increase in intravesical pressure was observed, & the micturition contraction was abolished after the parasympathetic denervation (Fig. 1).

(2) *Filling by stimulated urinary excretion* In 4 cats with an initial bladder volume of 5 ml of saline, glucose (5%) was administered i.v. at a rate of 70–25% of estimated blood volume per h. In previous experiments this was found to give maximum rates of urinary excretion (15 times the normal rate) (Klevmark 1974). After 9–27 min the animals responded with a smooth rise in the intravesical pressure which stabilized at a significantly higher level 6–16 min after discontinuation of the infusion (pressure accumulation phenomenon) (Fig. 2). Bladder volumes corresponded to an average filling rate of 4.4–9.3 HD suggesting considerably higher maximum rate of excretion. The intravesical pressure patterns observed after complete denervation during spontaneous and stimulated urinary excretion were identical to those previously described in the intact animal (Klevmark 1974).

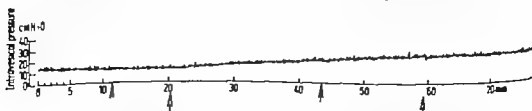
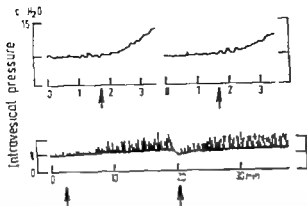


Fig. 5 Cat 3.0 kg. Changes in intravesical pressure during natural filling at high rates (stimulated urinary excretion) after complete extrinsic denervation. The initial bladder volume was 10 ml. Urinary excretion was stimulated with intravenous infusion of 5% glucose (see text). Start of infusion at arrow to the left. A gradual rise in pressure began after 9 min (second arrow). The infusion was stopped after 32 min (third arrow). The rise in pressure continued for 16 min, thereafter the pressure stabilized at a higher level (fourth arrow) (pressure accumulation phenomenon). Final volume was 47 ml giving an average rate of urinary excretion of 9.3 HD.



6. Intravesical pressure responses to repeated filling in the intact cat. Upper record: Cat 2.1 kg. Initial bladder volume 40 ml. Filling (at arrows) to the same final volume with 10 ml at the rate of 10 HD caused less increase in intravesical pressure the second time. Lower record: Cat 2.0 kg. Initial volume 40 ml. Filling (at arrows) to same final volume with 6 ml at the rate of 8.5 HD caused the same rise in intravesical pressure. Filling rate of 8.5 HD is within physiological range.

Intravesical pressure responses to repeated fillings in the intact cat

The following experiments were performed to study any possible reactions due to repeated stretching of bladder wall tissue. In 5 cats repeated filling from the infusion pump to equal initial volumes was performed using 10 ml of saline at 150 times the normal rate (150 HD). A smaller rise in pressure was observed the second time (Fig. 6, upper record). This finding was to some degree inconsistent when investigated from various initial bladder volumes. Repeated filling to equal final volume using 6 ml of saline at the rate of 8.5 HD caused the same increase in pressure (Fig. 6, lower record). This was a consistent finding from various initial bladder volumes as long as physiological rates of filling were used.

Discussion

Regulation of intramural tension (tonus) during controlled slow filling

In the present work regulation of bladder tonus was studied employing a technique which excluded the use of different physiological filling rates. When the rate of filling was low (about 1 HD), both the partially and the completely denervated bladder were filled without pressure increase as was the intact organ. After parasympathectomy the basal intravesical pressure was markedly elevated. However higher rates of filling gave intravesical pressure increases that were identical before and after sympathectomy or parasympathectomy.

The question of extrinsic nervous inhibition to counteract pressure increase at large bladder volumes (Edvardsen 1968 a, a.o.) has originated from cystometric studies using high and non-physiological filling rates. However the present study on denervated bladders has shown that, at large bladder volumes, there was no pressure increase to inhibit, as long as low physiological filling rates were used (Fig. 4). As in the intact cat (Klevmark 1974), pressure increase occurred at higher rates of filling, but at any volume. Thus, previous

cystometric methods, when used for the study of bladder tonus, do not illustrate his behaviour in the true collecting phase

Sympathetic inhibition of bladder tonus has been postulated, either regulated outflow from supraspinal levels (Gjono 1966) or through a spinal reflex (Edvardson De Groat and Lalley 1972, Sundin and Carlsson 1972, Sundin, Carlsson and Kock). However such inhibition was supposed to enhance the storage function by low intravesical pressure or bladder wall tension "at large volumes". In the present study sympathectomy did not change the pressure increase obtained during higher phasic filling rates irrespective of the volume present in the bladder.

Parasympathetic inhibition of bladder tonus from spinal or supraspinal levels has been suggested by several authors (Sherrington 1915, Langworthy and Kolb 1933, Langworthy and Heuser 1936, Kuru and Iwanaga 1966 *et al.*). On the other hand, Edvardson (1968 a) using conventional cystometry did not find efferent activity in the parasympathetic nerves during the collecting phase, findings that have been confirmed by De Groat and Ryall (1969). This is also in accordance with the present results where parasympathectomy did not change the intravesical pressure rise during higher rates of filling. However basal intravesical pressure was markedly elevated after parasympathectomy. This phenomenon was not observed when the sympathetic nerve supply had been eliminated. Hence it can be tentatively explained by increased sympathetic nervous activity. Endogenous α -adrenergic receptors have been located on postganglionic parasympathetic nerves in the bladder wall of the cat (De Groat and Saum 1972). Thus after extrinsic parasympathectomy this might be a site of increased sympathetic activity. An apparent change of β -adrenergic receptor response into an α -adrenergic receptor response has been demonstrated after parasympathetic bladder denervation in cats (Sundin and Dahlström, Norlén *et al.* 1976). Rise in intravesical pressure following parasympathectomy has been reported earlier in cats (Langworthy, Reeves and Tauber 1934) and in dogs (Lange, Whitehead 1951, Carpenter (1951). In a histological study found bladder hypertrophy in cats where parasympathectomy had been performed and infection prevented. Yet Carpenter and Root (1952) did not observe bladder hypertrophy after parasympathetic denervation in the absence of residual urine. However the present observation in experiments of the elevated basal pressure with unchanged pressure increase (compliance) during filling has not previously been reported. As an observation performed under isovolumetric conditions only this might be interpreted as "hypertonicity".

Intrinsic or paravesical reflex activity accounting for regulation of bladder tonus has also been postulated (Denny-Brown and Robertson 1933 b, Munro 1935). However use of pharmacologic blocking agents has failed to demonstrate such activity (Nesher, Lapides 1948, Carpenter and Root 1951, Tang and Ruch 1955, Kock and Pompeius 1961, Taira, Matsumura and Hashimoto 1969).

After complete extrinsic denervation the pressure rise at higher rates of filling was characterized by shorter periods (Fig. 3). This might be caused by the intrinsic short neurons described by Elbadawi and Schenk (1974). However it is difficult to see any importance of this finding for normal regulation of bladder tonus.

In the present investigation, controlled filling was performed at increasing rates with

physiological range. Such filling implied no measurable artifacts due to the procedure (Fig. 6), thus making interpretation of effects of denervation more reliable and physiologically relevant. Results presented herein support strongly the theory of a non-homogeneous basis of bladder tone.

Bladder adaptation during natural filling

Intravesical pressure patterns present during spontaneous and stimulated urinary retention in the intact animal (Klevmark 1974) were not changed by removal of the vesic nervous activity (Fig. 4 and 5). Thus, the "pressure accumulation phenomenon" (Fig. 5) must be due to bladder wall properties. The shape of the pressure curve, the smooth rise and decline, is due to the gradual change in rate of urinary excretion present during natural filling.

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Effect of Atropine and SC 15396 on Stimulated Gastric Acid Secretion in the Atlantic Cod, *Gadus morhua*

By

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Abstract

LITTE, B. Effect of atropine and SC 15396 on stimulated gastric acid secretion in the Atlantic cod, *Gadus morhua*. Acta physiol. scand. 1977 101 185-193

Gastric acid secretion was measured in swimming codfish surgically equipped with a catheter draining the stomach. Gastric acid secretion was stimulated by histamine (5 or 15 µg/kg h) or by carbachol (5 µg/kg h). Treatment with atropine (1 µmol/kg h) completely prevented the acid secretion induced by carbachol, but did not influence the secretion induced by histamine. Atropine had marked effects on the motor function of the stomach, and seriously reduced the volume draining from the stomach. Infusion of phenol red indicated that the decrease in volume was due to a decrease in recovery of ingested water. After SC 15396, "anticholinergic" significantly depressed acid secretion induced by histamine, and reduced carbachol-stimulated secretion, although the latter was statistically insignificant. The effects of SC 15396 as discussed with reference to the absence of receptors for gastrin related to gastric acid secretion in the codfish stomach.

Keywords: Antagonism, atropine, carbachol, fish physiology, gastric acid secretion, histamine

Codfish stomach responds to the i.m. injection of histamine or carbachol with the secretion of acid. Contrary to higher vertebrates, the codfish does not respond to pentagastrin, or to the injection of gastric mucosal extracts (Hölstén 1975). Thus, together with the unpublished observation, that bombesin (a tetradecapeptide with gastrin-releasing properties) is able to induce gastric acid secretion in the codfish, suggests that receptors for gastrin are present in the gastric secretory apparatus in this species.

In analogy with circumstances in many mammalian species, blocking of H_2 -receptors with metiamide counteracts the stimulatory actions of histamine and carbachol (Hölstén 1976). It remains an unsettled question whether this antagonism is due to some unknown property of metiamide, or to its H_2 -receptor blocking capacity. In the latter case, at least two possibilities must be considered, either histamine is a final common chemostimulator of the acid secreting cell (Code 1956), or are the receptors for histamine and cholinergic agonists (and gastrin) interdependent, as suggested by Grossman and Konturek (1974). Supporting their hypothesis is the depression by atropine of histamine-induced acid secretion commonly found in mammalian systems.

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Effect of Atropine and SC 15396 on Stimulated Gastric Acid Secretion in the Atlantic Cod, *Gadus morhua*

By

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Abstract

STERN, B. Effect of atropine and SC 15396 on stimulated gastric acid secretion in the Atlantic cod, *Gadus morhua*. Acta physiol. scand. 1977 101 185-193

Gastric acid secretion was measured in swimming codfish surgically equipped with catheter draining the stomach. Gastric acid secretion was stimulated by histamine (5 or 15 $\mu\text{g}/\text{kg}\cdot\text{h}$) or by carbachol (5 $\mu\text{g}/\text{kg}\cdot\text{h}$). Atropine (1 $\mu\text{mol}/\text{kg}\cdot\text{h}$) completely prevented the acid secretion induced by carbachol, but did not influence the secretion induced by histamine. Atropine had marked effects on the motor function of the stomach, and seriously reduced the volume draining from the stomach. Infusion of picrotoxin reduced the decrease in volume was due to decrease in recovery of ingested water. SC 15396, "antitachyarrhythmia" significantly depressed acid secretion induced by histamine, and reduced carbachol-stimulated acid secretion, although the latter was statistically insignificant. The effects of SC 15396 is discussed with reference to the absence of receptors for gastrin related to gastric acid secretion in the codfish stomach.

Key words: Antagonism, atropine, carbachol, fish physiology, gastric acid secretion, histamine

The codfish stomach responds to the Lm. Injection of histamine or carbachol with the secretion of acid. Contrary to higher vertebrates, the codfish does not respond to pentagastrin, to the injection of gastric mucosal extracts (Holstein 1975). This, together with the unpublished observation, that bombesin (a tetradecapeptide with gastrin-releasing properties) is to induce gastric acid secretion in the codfish, suggests that receptors for gastrin are absent in the gastric secretory apparatus in this species.

In analogy with circumstances in many mammalian species, blocking of H_2 -receptors by metiamide counteracts the stimulatory actions of histamine and carbachol (Holstein 1975). It remains an unsettled question whether this antagonism is due to some unknown property of metiamide, or to its H_2 -receptor blocking capacity. In the latter case, at least two possibilities must be considered, either histamine is a final common chemostimulator of the acid secreting cell (Code 1976), or are the receptors for histamine and cholinergic junctions (and gastrin) interdependent, as suggested by Grossman and Konturek (1974). Supporting their hypothesis is the depression by atropine of histamine-induced acid secretion commonly found in mammalian systems.

The present investigation was undertaken to further characterize the regulatory part of the codfish stomach. In particular the effect of an anticholinergic drug on histamine-stimulated acid secretion was considered important. It was also considered of interest to evaluate the effect of 2-phenyl-2-(2-pyridyl)-thioacetamide (SC 15396) on stimulated secretion. This drug was introduced as an antigestrin (Cook and Bianchi 1967), but subsequent work indicated that it was unspecific. The current idea, that the gastrin receptor is located in the secretory device of the codfish suggested the use of SC 15396 in this investigation.

Materials and Methods

Surgical procedure. Codfishes with a mean weight of 515 g (310–770 g) were used, and were prepared for collection of gastric effluence using a technique described in detail elsewhere (Holstein 1978). Briefly a polyethylene catheter (PE 700, Intramedic) was implanted in the pyloric part of the gut and passed through the intestinal and abdominal walls and finally through the trap of the splanchnic fraction collector. The water swallowed by the fish, mixed with the gastric secretions, was delivered to the catheter and divided into one-hour fractions.

Operations were carried out during the a.m., and the experiments during the next day. Before the animals were kept in storage aquaria, without food, for at least one week. The water temperature was 10°C.

During the progress of the study it became necessary to assess the recovery of injected phenol red, fishes were equipped with an extra catheter (PE 50), inserted through the stomach wall, approximately half way between esophagus and the pyloric orifice. Through that tubing, phenol red, dissolved in water was injected at a rate of 670 µg/h (0.62 ml/h).

Determination of acidity and atropine recovery. When possible, 2-ml aliquots of the effluent were titrated (Autotitrator assembly Radiometer Copenhagen) with 0.05 M sodium hydroxide to the pH of aquarium water. In cases of smaller samples, the whole sample was titrated following addition of 10 ml distilled water to make 2 ml.

The concentration of phenol red in each hourly fraction was determined by reading the optical density (503 nm) of 200 µl aliquots, acidified with 2.0 ml 0.1 M HCl. The recovery of phenol red was then calculated and considered to mirror the recovery of swallowed water and of secreted gastric acid.

Drugs. Atropine sulfate, Carbamylcholine chloride (Carbachol), Histamine dihydrochloride (SR 15396, Serierie). SC 15396 was dissolved in 50% polyethylene glycol (mol.wt. 370–430).

All drugs were administered as i.m. injections at a rate of 0.62 ml/h using a Braun Perfusor IV Infusion Pump. Doses given of histamine and carbachol refer to the salts.

Statistics. For each fish, the mean hourly acid output during drug (or placebo) administration was calculated. Then the means of these values were calculated for treatment and placebo groups respectively and compared by t-test (Program ST1-07, Statistical evaluation (two-sample test)) using a Texas Instruments SR 52 calculator. The difference was considered significant if a $P < 0.05$ was obtained (two-tailed test).

Results

1. Pretreatment with atropine. The injection of atropine 1 µmol/kg h during 3 h had no effect on the acid secretion induced by the subsequent administration of 15 µg/kg h histamine (Fig. 1, Table I). By contrast, atropine in the same dose completely abolished the secretory effect of 5 µg/kg h carbachol (Fig. 1, Table II).

Higher doses of atropine were used in combination with histamine only. Neither 5.1 or 25 µmol/kg h during 3 h was able to significantly alter the mean hourly acid output during the infusion of histamine (Fig. 2, Table II). However, both atropine doses decreased the unstimulated acid output (Fig. 2, Table III). It will be noticed from Fig. 2 (lower part) that also volume output decreased during atropine treatment.

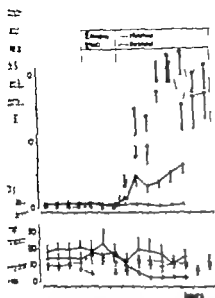


Fig. 1

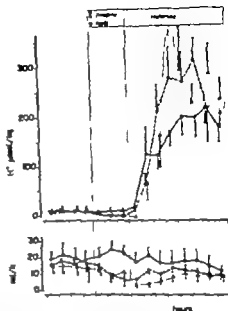


Fig. 2

Fig. 1. Effect of atropine (1 $\mu\text{mol/kg h}$) on gastric acid secretion stimulated by histamine (15 $\mu\text{g/kg h}$) or acid (5 $\mu\text{g/kg h}$). Means and \pm S.E. Number of animals is given in Table 1-11.

Fig. 2. Effect of atropine, 5 (\bullet) and 25 (\circ) $\mu\text{mol/kg h}$ on gastric acid secretion stimulated by histamine, 15 $\mu\text{g/kg h}$. Means and \pm S.E. See Table 1 for number of animals.

of the effect on unstimulated acid output, because in 12 of the 13 fishes, also the acidity was reduced (not illustrated).

Fig. 1 the results from 17 atropine-treated fishes are shown. In these fishes, atropine had no uniform effect on the volume output, but 4 additional experiments had to be discarded due to ceased water flow when atropine was instituted. For the same reason, 9 fishes treated with 5 and 25 $\mu\text{mol/kg h}$ atropine were excluded.

Table 1. Effect of pretreatment with atropine or BC 15396 on histamine-induced gastric acid secretion in the codfish.

No.	Dose $\mu\text{mol/kg h}$	Mean hourly acid output, $\mu\text{mol/kg h}$ Mean \pm S.E.	Number of animals	Significance
1-11	0	145.8 \pm 26.7	7	
1-11	1	120.7 \pm 15.4	2	0.4 P NS
1-11	0	159.1 \pm 18.2	7	
1-11	5	199.8 \pm 31.6	7	0.3 P NS
1-11	25	202.7 \pm 28.6	4	0.2 P NS
1-11	0	120.8 \pm 22.7	7	
1-11	10	118.4 \pm 18.9	7	0.9 P NS
1-11	0	221.2 \pm 30.4	7	
1-11	50	93.0 \pm 19.2	9	0.001 < P < 0.01

TABLE II Effect of pretreatment with atropine or SC 15396 on carbachol-induced gastric acid secretion in the codfish

Agent	Dose $\mu\text{mol/kg h}$	Mean hourly acid output, $\mu\text{mol/kg h}$ Mean \pm S.E.	N mber of animals	Significance
Atropine	0	42.7 \pm 6.8	10	P < 0.001
	1	5.5 \pm 2.4	9	
SC 15396	0	61.3 \pm 10.9	9	0.2 < P NS
	10	43.7 \pm 6.7	10	
SC 15396	0	52.3 \pm 6.9	10	0.1 < P NS
	50	33.9 \pm 9.6	12	

Carbachol caused an immediate decrease in water output; this effect was prevented by atropine pretreatment (Fig. 1) confirming the suggestion that the decrease was mediated by muscarinic receptors in the stomach smooth muscles (Holstein 1976). In Fig. 1 ten or (saline-carbachol) fishes are reported, additionally 7 were discarded due to complete stop of water flow during carbachol administration.

2. Atropine during histamine injection In these experiments the fishes received 50 μg histamine to initiate secretion and, at the same time, the infusion of 15 $\mu\text{g/kg h}$ of histamine was started. Atropine was given for 3 h, beginning after 5 h of histamine administration. Two doses of atropine were used, 2 and 5 $\mu\text{mol/kg h}$. The results were indistinguishable and are reported together. In 3 of the 17 fishes prepared for these expts., water output stopped as soon as atropine was instituted. In the remaining, a more or less marked decrease in water output took place and sometimes water flow stopped for one h, but was then resumed. From the measured parameters (decreasing volume and increasing acidity), a decrease in acid output was calculated during atropine treatment (not illustrated).

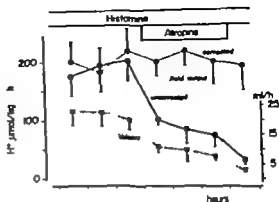
The expt. was repeated on 11 fishes prepared for infusion of phenol red as described in *Materials and Methods*. Of these, 12 immediately stopped delivering water when atropine was injected, and 2 were apparently unaffected. In the remaining 4 the anticholinergic caused a decrease in volume output, accompanied by an apparent inhibition of acid output (Fig. 3). However, the recovery of phenol red also decreased, and when the acid output was corrected accordingly, no inhibition could be demonstrated.

The recovery of phenol red for the three h preceding atropine infusion was calculated.

TABLE III. Effect of atropine on unstimulated gastric acid secretion in codfish.

Mean hourly acid output during 3 h before treatment, $\mu\text{mol/kg h}$		Treatment Atropine $\mu\text{mol/kg h}$	Mean hourly acid output during 3 h treatment, $\mu\text{mol/kg h}$	
Mean \pm S.E.	F		Mean \pm S.E.	P
13.3 \pm 3.4	0.5 < P NS	0	14.2 \pm 3.6	0.02 < P < 0.05
10.7 \pm 1.9		5	3.1 \pm 0.8	
17.6 \pm 6.2	0.5 < P NS	5	3.8 \pm 1.2	0.02 < P < 0.05

Effect of atropine ($2 \mu\text{mol/kg h}$) imposed upon an injection of histamine ($15 \mu\text{g/kg h}$), on gastric acid secretion. 30 min before atropine, $30 \mu\text{g/kg}$ histamine administered to start the secretion. Phenol red was infused into the ch during the whole sequence.

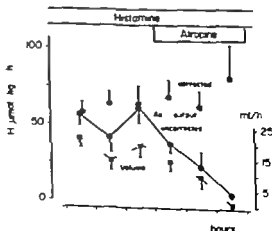


values (18) and found to be 91.4 ± 3.0 , 86.1 ± 3.1 and 91.0 ± 1.8 (mean \pm S.E.) for the 3 h respectively.

Alternatively the same result was obtained with a lower dose of histamine, as shown in 4. Four fishes out of 9 maintained the delivery of water despite the fact that the atropine was reduced to $0.5 \mu\text{mol/kg h}$.

Although the recovery of phenol red decreased when atropine was instituted, the concentration of the dye in the gastric effluence sometimes increased, sometimes decreased. As the effect of atropine on water intake is inconsistent.

Pretreatment *nk* SC 15396. Two doses of this drug were used. 10 and $50 \mu\text{mol/kg h}$. Lower dose had no effect on the histamine-induced acid secretion (Fig. 5, Table I), but reduced the carbachol stimulated secretion to 71% of the control level. This effect, however, was not statistically significant (Fig. 5, Table II). The higher rate, $50 \mu\text{mol/kg h}$, reduced the histamine induced secretion to 42% of control level (Fig. 6, Table I) and the



4 See legend to Fig. 3. I. These experiments, secretion was stimulated by $20 \mu\text{g/kg}$ plus $5 \mu\text{g/kg h}$ histamine, respectively, $0.5 \mu\text{mol/kg h}$.

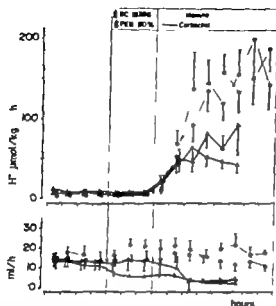


Fig. 5

Fig. 5 Effect of SC 15396, 10 $\mu\text{mol/kg h}$, on gastric acid secretion stimulated by histamine (15 $\mu\text{g/kg h}$) or carbachol (5 $\mu\text{g/kg h}$). Means and 1 S.E. See Table I II for number of animals. PEG polyethylene glycol.

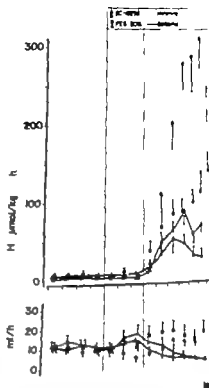


Fig. 6

Fig. 6 See legend to Fig. 5. In these experiments, 50 $\mu\text{mol/kg h}$ SC 15396 was infused.

carbachol-induced secretion to 65% (Fig. 6, Table II). Again, the effect on carbachol-induced secretion was not statistically significant.

4 SC 15396 during histamine administration When SC 15396 was superimposed upon infusion of histamine, the previously increasing acid output decreased (Fig. 7). SC 15396 had no effect on the water output, neither did it influence the recovery of phenol red when infused during these experiments.

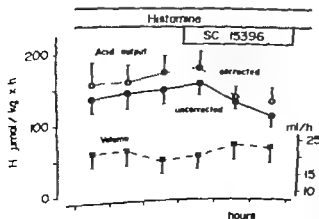


Fig. 7 Effect of SC 15396, 50 $\mu\text{g/kg h}$, on gastric acid secretion stimulated by histamine, 15 $\mu\text{g/kg h}$. 5 h SC 15396, 50 $\mu\text{g/kg h}$ histamine injected. Phenol red was infused in stomach during the whole experiment. Means and 1 S.E. ($n=7$).

Discussion

SC 15396 ("antigastrin") is considered to be a general inhibitor of gastric acid secretion. and Thompson (1968) found that the drug antagonized the acid-stimulating effects of gastrin, insulin, and histamine. Kahlson *et al.* (1968) demonstrated inhibition of H^+ - and histamine-induced secretion and concluded that the drug reduces the responsiveness of the parietal cell to histamine. Such reduced sensitivity could be transmitted by a specific antigastrin—as remarked by Thompson *et al.* (1968)—provided that the activity of the H^+ receptor is needed for full responsiveness of the parietal cell. However this view is to reconcile with the finding of Kahlson *et al.* (1968), that the gastrin-induced elevation histamine forming capacity is increased by SC 15396, and the demonstration by Caren (1969) and Håkanson and Ljefberg (1971) that rat gastric histidine decarboxylase (Hd) stimulated by SC 15396. According to Håkanson and Ljefberg (1971), Hd is stimulated by H^+ which is released as a result of increased intral pH following SC 15396-blockade of secretion. Thus, the view that SC 15396 is a specific antigastrin can only be maintained if receptors for gastrin related to gastric acid secretion are different from those mediating induction of Hd. Such differentiated specificity among receptors for gastrin was referred to by Caren *et al.* (1969), as a possible explanation of the failure of secretin to counteract antigastrin-induced activation of Hd.

The result of the present investigation confirms the conclusion that SC 15396 is an H^+ -specific inhibitor of gastric acid secretion, because histamine-induced secretion was depressed. In a system devoid of gastrin receptors related to acid secretion, a specific antigastrin should *a priori* be without effect.

As expected, atropine is a powerful inhibitor of carbachol-induced gastric acid secretion in the cod. A dose-rate of $1 \mu\text{mol/kg h}$ (0.68 mg/kg h) during 3 h, completely abolished response to a subsequent 6 h-injection of carbachol. This rate, and even the 5- or 25-fold increase did not depress the secretion elicited by a subsequent injection of histamine. However evaluation of this result is complicated by the fact that some fishes stopped the delivery of water when atropine-treated. Thus, these fishes appeared to be more sensitive than the others concerning the motor activity of the stomach. This might also apply to the secretory effect. When atropine was superimposed upon an infusion of histamine, the motor effects appeared exaggerated, but from few fishes decreasing volume output was maintained for several hours. The acid output during these hours decreased, but again the evaluation is complicated, this time by concomitant increase in acidity. The decrease in acid output reflects real inhibition if the increased H^+ -concentration is the result of an atropine-induced reduction in drinking rate. It is equally likely however that atropine interferes with gut motility in a manner which decreases the recovery of ingested water. In that case, the decreased acid output is an artifact, resulting from loss of water and acid, presumably through esophagus. The latter interpretation is favoured by the results obtained with infusion of phenol red during atropine treatment: there is a decrease in water recovery and following correction, no inhibition of histamine-induced secretion could be demonstrated. Admittedly decrease in recovery of phenol red could be the result of accumulation of fluid in a dilating stomach. If pooling takes place during atropine treatment, the corrected output values are

underestimated and therefore do not obviate the conclusion that no inhibition takes place. This demonstration, that acid secretion is unaffected by atropine despite conspicuous effects on motor functions makes the results with atropine-pretreatment reliable, and it is concluded that in the codfish, histamine-induced gastric acid secretion is not amenable to inhibition by atropine.

In mammals, the inhibitory effect of atropine on histamine-stimulated secretion is of competitive type (Konturek *et al* 1968, Borg and Ernäs 1970, Hirschowitz *et al* 1972, 1973). In the present investigation two doses of histamine were used. Of these, at least one is maximal, refuting the objection that a possible competitive inhibition was surpassed by a supramaximal dose of histamine.

Grossman and Konturek (1974) when presenting the hypothesis of interdependence of histamine and acetylcholine receptors on the parietal cell postulate that, in the dog, "blocking the acetylcholine receptor only weakly suppresses the histamine receptor whereas blocking the histamine receptor moderately inhibits the acetylcholine receptor". From the result of the present work, it seems to be no reason to suggest any suppression of the histamine receptor when the cholinergic one is blocked. The reverse, however, may still be true: metiamide potentially antagonizes the stimulating effect of carbachol on gastric acid secretion in the codfish (Holstein 1976).

The existence of a cholinergic link in the regulation of gastric acid secretion in the codfish is suggested by the stimulating effect of carbachol and by the blockade of that action by atropine. Also the basal secretion was depressed by atropine, but in that case, the site of interaction is not necessarily peripheral. It can not be excluded that basal secretion is regulated by a mechanism which is centrally cholinergic, but peripherally non-cholinergic. However, a vagal tone does not seem to be a prerequisite for atropine to inhibit histamine-stimulated acid secretion (Konturek *et al* 1968).

The finding that atropine is without effect on acid secretion stimulated by histamine indicates, that compared to mammals, the regulatory mechanism of the codfish stomach is less complex. The result of a previous work in which pentagastrin was found to be ineffective as a secretory stimulant (Holstein 1975) also indicates a less complex regulation. Whether this is a primitive condition, or a specialized one, is an open question.

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Fatigue and EMG of Repeated Fast Voluntary Contractions in Man

By

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Abstract

NILSSON, J., P. TESCH and A. THORSTENSSON. *Fatigue and EMG of repeated fast voluntary contractions in man*. *Acta physiol. scand.* 1977 101 194-198

A fatigue test consisting of repeated fast maximal contractions of the left quadriceps muscle in a apparatus was performed by 10 healthy male subjects (19-25 yrs). EMG signals were recorded from the surface of the left vastus lateralis muscle, from which also torques were obtained for muscle fatigue test. Only minor changes were observed in the EMG variables despite a decrease in muscle force after 1 series of peak torque, a 4% and power a, about 40% of initial values after 100 contractions. The electromyograph obtained positive correlation between the increase in EMG/torque ratio and the individual percentage of fast twitch (FT) muscle fibres indicated that local factors in the muscle in the FT fibres, were causing the development of fatigue during repeated dynamic contractions and power outputs.

Changes occurring in the EMG have often been used as a means to investigate mechanisms in human skeletal muscle. So far the studies have mainly been concerned with fatigue during isometric loading. Still there are considerable disagreement about the behaviour of the EMG during fatigue. Merton (1954) found no changes in EMG during repeated maximal contractions and therefore he ascribed fatigue entirely to the processes in the muscle itself. In contrast Stephens and Taylor (1972) found that the EMG declined with fatigue during maximal voluntary isometric contraction. During the first phase (about 1 min) the force fell to about 40% and the EMG decreased with the same rate. In the second phase the force fell relatively faster than the EMG and the EMG remained at approximately steady level at about 45% of initial values. The authors concluded that fatigue of the muscular junction (mainly of the high threshold motor units), was more marked initially whereas contractile element fatigue (mainly confined to the low threshold motor units), became more marked later. (High and low threshold motor units correspond to units with fast twitch, FT type II and slow twitch, ST type I muscle fibres, Burke and Edgerton 1975.)

Recently Thorstensson and Karlsson (1976) developed a fatigue test in which the force was measured as peak torque during repeated maximal isokinetic contractions.

motion speed. Also in this test the force decreased rather rapidly to reach a plateau after 90 s (corresponding to approximately 45 contractions). The decline in force was shown to be positively correlated to the proportion of FT fibres in the contracting muscle. It is therefore of interest to apply an EMG analysis also on repeated fast dynamic motions to get an indication about the fatigue mechanisms involved. In addition, the relations between peak torque, power and work, as well as peak and integrated EMG were studied at different levels of fatigue and with respect to the distribution of muscle fibre types in the contracting muscle.

Material and Methods

Twelve habitually active male subjects volunteered for the study. Their mean age, height, and weight were 22 ± 3 yrs, 180 ± 3 cm, and 70.3 ± 5.5 kg, respectively. The equipment and test protocol have been described in detail earlier (Thorsen 1974, Thorsen and Karlsson 1976, Thorsen 1976). Briefly the subjects performed 100 s of extensions of the left knee joint at a constant angular velocity of $180^\circ/\text{s}$ (3.14 rad/s) at 30 contractions per minute. The range of motion was from 90 to 0° knee angle, i.e. each contraction lasted 0.5 s and the passive return approximately 0.7 s. The coefficient of variation for the peak torque with 30 contractions was 3.2 (Thorsen and Karlsson 1976).

Peak torque was defined as the highest point on the torque curve.

Work was taken as the area under the torque curve and measured with a planimeter or was calculated as work divided by the time for the contraction.

Mean values of contractions No. 1-3, 23-25, 48-50, 73-75, and 94-100 were taken for comparison. In all instances care was taken to ensure that the subjects did exert their greatest possible effort.

Muscle fibre classification. Muscle biopsies were obtained from the vastus lateralis muscle with needle techniques (Bergström 1962). Muscle fibres were classified as FT or ST after histochemical staining with alkaline ATPase (method originating from Padykula and Herman 1955).

EMG-recording. The EMG activity was recorded from the contracting vastus lateralis muscle. EMG signals from different portions of the engaged quadriceps muscle showed that the vastus lateralis was suitable for the whole muscle group. Surface electrodes were used to pick up the activity. The amplified EMG signals were rectified and filtered by a single lag filter of 120 ms time constant. The filtered signal was displayed on a UV-recorder (Honeywell Visucorder 2012) along with the corresponding torque signal.

Peak EMG was defined as the highest point on the rectified and filtered EMG curve.

Work was taken as the area under the rectified and filtered EMG curve as measured by planimeter.

Ordinary statistical methods were used to calculate means, standard errors of the means, and linear correlation coefficients (r). Differences were tested for significance with the Student's t -test.

Results

Mean values for peak torque, work and power averaged 140.8 N.m , 121.1 J and 272.2 W respectively. Peak torque was well correlated with work ($r = 0.80$, $p < 0.01$) and power ($r = 0.81$, $p < 0.001$). A positive correlation ($r = 0.75$ - 0.81 , $p < 0.01$) was also observed between FT fibres and these variables during the initial contractions.

Correlation coefficients (r) for peak EMG and IEMG during the initial contractions were 1.39 ± 0.11 and 1.26 ± 0.11 in arbitrary units. The correlation coefficient between these measures was 0.93 ($p < 0.001$).

The changes occurring in the measured muscular force and EMG variables with increasing fatigue are demonstrated in Fig. 1. Peak torque, work and power values declined with each contraction. Thereafter these variables tended to plateau at a

Fatigue and EMG of Repeated Fast Voluntary Contractions in Man

By

J NILSSON P TERCH and A. THORSTENSSON

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Abstract

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A fatigue test consisting of repeated fast maximal contractions of the left quadriceps muscle in an apparatus was performed by 12 healthy male subjects (19-25 yrs). EMG signals were recorded from the surface of the left vastus lateralis muscle, from which also biopsies were obtained for muscle fibre conduction velocity. Only minor changes were observed in the EMG variables despite a decrease in muscle performance, in terms of peak torque, work and power to about 50% of initial values after 100 contractions. The concomitantly obtained positive correlation between the increase in EMG/torque ratio and individual percentage of fast twitch (FT) muscle fibres indicated that local factors in the muscle, in the FT fibres, were causing the development of fatigue during repeated dynamic contractions and power outputs.

Changes occurring in the EMG have often been used as a means to investigate mechanisms in human skeletal muscle. So far the studies have mainly been for fatigue during isometric loading. Still there are considerable disagreement over the behaviour of the EMG during fatigue. Merton (1954) found no changes in EMG during sustained maximal contractions and therefore he ascribed fatigue entirely to the conduction processes in the muscle itself. In contrast Stephens and Taylor (1972) found the EMG declined with fatigue during maximal voluntary isometric contraction. During the first phase (about 1 min) the force fell to about 50% and the IEMG decreased with the same course. In the second phase the force fell relatively faster than the IEMG and reached an approximately steady level at about 25% of initial values. The authors concluded that muscular junction fatigue, mainly of the high threshold motor units, was most marked initially whereas contractile element fatigue, mainly confined to the low threshold motor units, became more marked later. (High and low threshold motor units presumably correspond to units with fast twitch, FT type II and slow twitch, ST type I muscle fibres, Burke and Edgerton 1975.)

Recently Thorstensson and Karlsson (1976) developed a fatigue test in which the force was measured as peak torque during repeated maximal isokinetic con-

Discussion

results of the present study confirmed earlier findings with respect to the torque measurements (Thorstensson and Karlsson 1976). Peak torque values declined progressively to reach a level at about 50% of initial values after approximately 50 contractions. The relative decline in peak torque was found to be related to the percentage of FT fibres in the contracting muscle. Close agreement was found between peak torque, work and power in these tests.

However, despite the marked decline in torque no corresponding changes were observed in the EMG. If anything, there was a slight increase in the EMG output during the first 25 contractions. One possible reason for this might be that the subjects did not exert maximal efforts in the initial contractions of the fatigue test. To control this possibility an additional test was performed under identical conditions. The subjects were asked to perform only single maximal efforts at the corresponding angular velocity (180 degrees/s). In this case the measured variables tended to reach somewhat higher values (0-7%), but a statistically significant difference ($p < 0.05$) was seen only for work.

The present results were in contrast to those of Stephens and Taylor (1972), who found a decrease in force during the initial phase of maintained maximal isometric contraction of the first dorsal interosseous muscle of the hand that was of the same order of magnitude as in the present study. However, they found a concomitant and proportional decrease also in the EMG, which pointed at the neuromuscular junction (NMJ) as the site of fatigue. Thus, although the course of fatigue appeared similar, the cause of fatigue seemed to be different in the two experimental situations. Circumstances such as differences in the size and structure of the muscles investigated, number and type of motor units recruited in maximal isometric and fast dynamic contractions as well as the intermittent character of the dynamic test have to be considered when evaluating this discrepancy.

The present findings of an unchanged or even increased level of EMG activity with fatigue could theoretically be explained by additional recruitment of motor units (with less ability to produce force) as the number of active FT units decreased due to NMJ fatigue. However, the most likely explanation appeared to be that some local factor in the muscle itself was the cause of the fatigue and not the neuromuscular junction. The correlation found between the increase in EMG/torque ratio with fatigue and percentage of FT fibres indicated that the detrimental factor for the decrease in force output was localized mainly in the FT fibres during the successive drop out of this type of fibres. The positive correlation between the percentage of FT muscle fibres and the increase in lag phase between EMG activity and muscle force output with fatigue would also be compatible with this hypothesis. Possible causes for fatigue could be exhaustion of the elastic component, a lack of immediately available substrates and accumulation of metabolites, e.g. lactic acid. The latter possibility was supported by preliminary results from lactate determinations on single muscle fibres in experiments with the present protocol (Tesch *et al.* to be published).

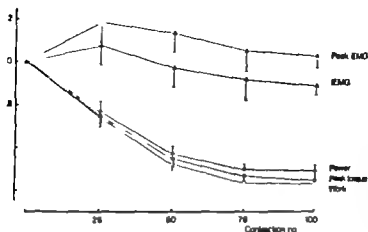


Fig. 1. Means \pm SE of measured variables during the course of fatigue. The values were arbitrarily set. (For absolute values, see text.)

level corresponding to about 45–55% of initial values. Significant correlations were observed between relative decline in peak torque, work and power with 50 contractions and $r = 0.75$ ($p < 0.01$), $r = 0.64$ ($p < 0.02$) and $r = 0.73$ ($p < 0.01$), respectively.

No corresponding decrease was observed in the EMG variables (Fig. 1). In contrast, peak EMG values for the 25th and the 50th contraction were higher ($p < 0.05$) than initial. A similar tendency (n.s.) was present for IEMG after 25 contractions. The daily EMG were not correlated to % FT.

Both peak EMG per unit peak torque and IEMG per unit work demonstrated a progressive increase from the initial to the 75th contraction, after which an apparent steady state was reached. This level corresponded to about 2–2.3 times initial values. The relative increase in these ratios with 50 contractions was correlated to the individual percentage of fibres. The correlation coefficients were $r = 0.84$ ($p < 0.001$, Fig. 2) and $r = 0.78$ ($p < 0.01$), respectively.

The time lag between the first action potential in the EMG and the corresponding recording (= the point when the pre-set velocity was reached) increased progressively from 95 ± 3 ms initially to 115 ± 5 ms at the 50th, and 121 ± 5 ms at the 100th contraction, respectively. The initial values as well as the relative increase in time lag was correlated with $r = -0.68$ ($p < 0.02$), and $r = -0.73$ ($p < 0.01$), respectively.

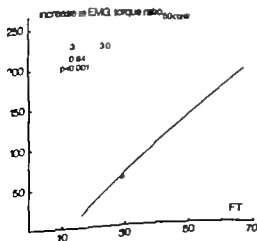


Fig. 2. Relationship between the relative increase in peak EMG/peak torque ratio with 50 contractions and the percentage of FT fibres in the vastus lateralis muscle (see text and Fig. 1).

Discussion

Results of the present study confirmed earlier findings with respect to the torque measure- (Thornstrom and Karlsson 1976). Peak torque values declined progressively to reach a level at about 50% of initial values after approximately 50 contractions. The relative change in peak torque was found to be related to the percentage of FT fibres in the contracting muscle. Close agreement was found between peak torque, work and power in these tests.

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Frequency-Dependence of ³H-Noradrenaline Secretion from Human Vasoconstrictor Nerves: Modification by Factors Interfering with α - or β -Adrenoceptor or Prostaglandin E₂ Mediated Control

By

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Abstract

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Isolated superfused field stimulated human omental arteries and veins, preincubated with ³H-(-)-noradrenaline (NA) were used to study the frequency dependence of NA secretion and of the mechanisms for local feedback control. ³H-NA secretion per shock was found to be biphasically - a simple hyperbolic rise of the stimulation frequency from 1 to 30 Hz, as long as secretion was restricted by prostaglandin (PGE₂) to the extent of restriction, or during facilitation, ³H-NA secretion per shock reached its maximum at 10 Hz and then declined at 30 Hz, indicating "overload" in some link in the secretory mechanism. ³H-NA secretion was depressed by exogenous NA and by PGE₂, and enhanced by isoprenaline, tolazoline and by blockade of PGE₂ formation. Most of these effects were reversibly related to the release frequency. Attempts are made to study interactions between the different control mechanisms in order to evaluate possible in vivo consequences of disturbance of adrenergic neurotransmission by interference with the local control of the secretory mechanism.

A certain amount of noradrenaline (NA) secreted from adrenergic nerve terminals, on arrival at the target, is not constant but subject to complex local control. It is mainly restricted by an α -adrenoceptor mediated mechanism (Häggendal 1970, Farnébo and Hamberger 1971, Kurpekar and Palg 1971, Starke 1972, Enero *et al.* 1972), and by local formation of prostaglandin E₂ (PGE₂, Hedqvist and Brundin 1969, Hedqvist 1970, Wachtman and Wennmalm 1971, Wennmalm 1972), and it may in certain tissues and vessels be augmented by a β -adrenoceptor mediated mechanism (Adler-Graschinsky and Langer 1975, SjöLärme 1975). These different control mechanisms have also been found to operate on the secretion of ³H-NA from the adrenergic nerves of isolated human omental blood vessels (SjöLärme and Gripe 1973, SjöLärme 1975a, SjöLärme and Brundin 1975a, b, 1976a, b).

In many tissues and species the amount of NA secreted per impulse is also dependent on the frequency of nerve stimulation (Brown and Gillespie 1957 for reviews see Kir 1975 Stjärne 1975 b). In a previous study of the relationship between the 'classically mediated' local control and that exerted by variation in the frequency of nerve stimulation (in guinea pig isolated vas deferens), it was found that the efficiency of both the frequency-dependent and PGE₂-independent α -adrenoceptor mediated control of ³H-NA secretion was inversely related to the frequency of nerve stimulation (Stjärne 1973).

In the abovementioned previous work concerning local control of the secretion of ³H-NA from the vasoconstrictor nerves of human omental blood vessels a frequency of 1 Hz was used, in order to ensure that stimulation was within the physiologically significant range. The present study was carried out to clarify the frequency dependence of the process of ³H-NA secretion in these nerves as well as the frequency dependence of the various mechanisms for its local feedback control.

Material and Methods

The biopsy specimens (3–6 cm pieces of arteries and veins from the omentum, of an external diameter about 1.5 mm) were taken from 33 normotensive patients (30 female, 3 male, age range 16–67, median age 37) undergoing abdominal surgery for various causes, under routine general anaesthesia (most cases with halothane/oxygen/nitrous oxide/curarine). Premedication always included 0.5 mg, in addition to promethazine 25 mg or pethidine 25–75 mg s.c. In the 7 cases of Caesarean section (Syntocinon) 2 IU was administered i to the myometrium.

The method for labelling the neural stores with ³H-(+)-NA and for superfusion of the preparations was identical to that previously described (Stjärne and Brundin 1976 a). The electrode arrangement and stimulation parameters were different however. In the present study the intramural nerves were stimulated via two platinum ring electrodes, one at the top and one at the bottom of the preparation (loop diameter 3 mm, distance between electrodes 5 cm). Stimulation was biphasic, and the shocks are delivered by a Grass S44 stimulator at a voltage of 120 V and a duration of 0.3 ms. During each stimulation period shocks were applied at frequencies ranging from 1 Hz to 30 Hz.

Since the medium contained 0.6 μ M desipramine and 10 μ M normetanephrine, tissue binding of ³H-NA should be minimized (Iversen 1971). The secretion of ³H-NA evoked by nerve stimulation was expressed as Δt

$$\Delta t = \frac{\text{Evoked rise in efflux of } ^3\text{H-NA}}{\text{Calculated total } ^3\text{H-NA in the tissue at beginning of stimulation}}$$

Nerve stimulations were applied with 16 (or 4 ring washes 3) ml intervals. Drugs, at the concentrations given in the paper, were added to the medium 8 ml prior to the next stimulation. The drugs did not alter the resting efflux of ³H-NA.

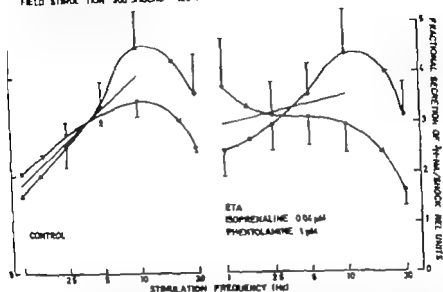
The following drugs were used: Isoprenaline (L-isoprotrenol-D-bitartrate, Sigma), propranolol, phenolamine (Hissle-Che-Geigy), noradrenaline bitartrate (Sigma), prostaglandin E₂ (Aspirin), irreversible inhibitor of PGE₂ synthesis (Downing et al 1970) and 11,14-eicosatetraynoic acid (ET) received as a gift from Professor B. Samuelsson at the Department of Biochemistry, Karolinska Institute.

Statistical evaluation of the results was by Student's *t* test. The figures given in the paper are means \pm S.E.

Results

A. Control values for Δt . The average absolute value for Δt per shock during the 23 control stimulations at 1 Hz applied at the start of each experiment was 5.63 ± 0.10 (n=122) of the total store of ³H-NA. All subsequent values for Δt obtained stimulation either in the sequence 1, 2.5, 5, 10 and 30 Hz, or 1, 5, 10, 20, 30 Hz or

FREQUENCY DEPENDENCE OF Δ -NA SECRETION IN HUMAN ORIENTAL BLOOD VESSELS.
EFFECT OF REVERSING SEQUENCE OF FREQUENCIES OF STIMULATION (ARROWS)
FIELD STIMULATION 300 SHOCKS 120 V 8.3 ms



1. Hysteresis in values for Δ in stimulation at rising or falling frequencies (1-30 Hz). Points and vertical lines and ranges of 2 observations. Regression lines show estimated slopes at 1-10 Hz. 'peak' Δ in the absence of drugs (0.6 μ M desipramine and 10 μ M noroctadecaprine present throughout). 'left panel' Δ at highest degree of facilitation in the study. Ordinate: Note differences between left and right ordinates (same relative units, see the Text).

not addition of drugs, were normalized in relation to the average value for Δ during 'initial control' stimulations at 1 Hz in that same experiment.

1. Hysteresis in values for Δ : In the absence of drugs Δ per shock increased with the frequency of nerve stimulation, from 1 Hz to 10 Hz, but declined at 30 Hz, both when stimulations were applied in the order 1-2.5-5-10 and 30 Hz and when the sequence was reversed (Fig. 1 left panel). In spite of the considerable hysteresis illustrated in Fig. 1 there can be no doubt that Δ increased with the frequency of nerve stimulation from 1 to 10 Hz, in the control experiments. The hysteresis was regarded as an indication of fatigue in the secretory mechanism. Therefore only one series of frequencies was studied in each experiment (after the 2-3 initial control stimulations at 1 Hz), either in the sequence 1-2.5-10 and 30 Hz or in the reverse order. The results shown (except in Fig. 1) represent means \pm S.E. of an equal number of experiments with rising and falling frequencies of nerve stimulation. The rapid onset of 'fatigue' in the secretory mechanism, on stimulation in the presence of drugs very markedly raising Δ (Fig. 1 right panel shows the type of experiments here the highest levels of Δ were reached), made it very difficult to draw any reliable conclusions from this type of approach concerning the effects of the drugs on the secretory mechanism at the different frequencies of stimulation. Because of this, additional experiments were carried out where only 2 stimulations were applied after the initial 2-3

EFFECT OF DRUGS ON ^3H -NA SECRETION
HUMAN ORIENTAL BLOOD VESSELS
FIELD STIMULATION 300 SHOCKS 120 V 0.3 ms

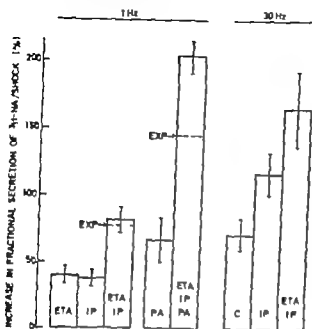


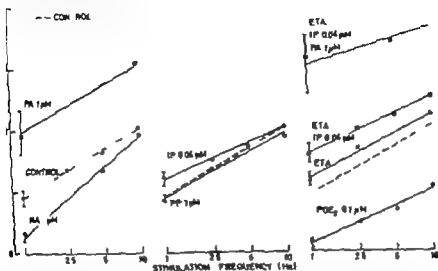
Fig. 1. Effect of drugs on Δt per shock stimulation for 2 periods (1 and 30 Hz) of Results. B. Overall mean Δt during control stimulation at 1 Hz: 40 ± 3 S.E. Asterisks indicate $p < 0.05$, $p < 0.001$. Abbreviations: IP = isoproterenol $0.04 \mu\text{M}$; PA = phentolamine $1 \mu\text{M}$; expected. Left panel: Drug-control results at 1 Hz. ETA and IP alone: $p < 0.001$ to controls. ETA+IP: $p < 0.001$ to controls or IP alone. PA: $p < 0.001$ to controls. ETA+IP+PA: $p < 0.001$ to controls. Right panel: Stimulation at 30 Hz. Δt over control at 1 Hz: $p < 0.05$ to control at 1 Hz. IP: $p < 0.001$ to control at 30 Hz. ETA+IP: $p < 0.001$ to control at 30 Hz.

control stimulations, one at 1 Hz and one at 30 Hz, either with or without additional different drugs (Fig. 2).

C. *Frequency dependence of the secretory mechanism.* The values for Δt per shock entirely increased with the frequency of nerve stimulation from 1 to 10 Hz, with or without addition of drugs (Fig. 3), but declined on stimulation at 30 Hz (Fig. 1), except in experiments where PGE₂ was added to the medium (Fig. 4). This agent very markedly depressed Δt per shock at all frequencies (see Fig. 3). The straight line obtained in a semilog plot of the PGE₂-depressed values for Δt as a function of stimulation frequency from 1 to 30 Hz (Fig. 4) indicates that the efficiency of the secretory mechanism tended to increase hyperbolically with the total duration of depolarization per unit time. The decline in Δt per shock seen on stimulation at 30 Hz, in the other experiments where the secretory mechanism was not depressed (control experiments, or experiments in presence of drugs enhancing Δt) was therefore probably not due to failure of propagation of nerve impulses at 30 Hz, but rather to beginning failure of some link in the secretory mechanism (see also Fig. 6).

D. *Frequency-dependence of α -adrenoceptor mediated feedback control of Δt .* As shown in Fig. 3 $1 \mu\text{M}$ NA very markedly depressed Δt on stimulation at 1 Hz (by 67%, $p < 0.001$). The depressing effect declined with increasing frequencies of stimulation (Fig. 5), and was not statistically significant even at 5 Hz ($p > 0.05$). Exogenous NA therefore changed the slope of the regression line shown in Fig. 3 (from 1.013 for the control to 1.014). Conversely $1 \mu\text{M}$ phentolamine appeared to enhance Δt at all frequencies (although hysteresis caused a considerable scatter of values making significance evaluation difficult) and therefore did not tend to alter the slope of the regression line showing the

FREQUENCY DEPENDENCE OF 2H-NA SECRETION IN HUMAN ORIENTAL BLOOD VESSELS
EFFECTS OF INTERFERENCE WITH ITS FEEDBACK CONTROL
FIELD STIMULATION 300 SHOCKS 120 V 0.3 ms



1. Regression lines: Frequency dependence of ΔI in controls. Effects of drugs on frequency dependence: Bars: Average S.E. for ΔI at 1-10 Hz in controls or in the presence of drugs. Abbreviations: PA = isoprenaline; IP = isoprenaline; PP = propranolol. Left panel: Effects of α -agonist and -antagonist. Right panel: Effects of β -agonist and -antagonist. Right panel: Effects of exogenous PGE_2 and of ETA (to block action of PGE_2). Effects of ETA β -agonist or ETA + β -agonist -antagonist. Ordinate: Same as in Fig. 1.

coincide of ΔI in Fig. 3 (slope with phenolamine 1.0662). Phenolamine thus shifted regression line describing the frequency dependence of ΔI per shock upwards by 73% actively to control values at each frequency the enhancing effects of phenolamine on ΔI were inversely related to the frequency of stimulation (Fig. 5). In the type of experiments shown under B (post stimulation period only at 1 and 30 Hz, Fig. 2) 1 μM phenolamine increased ΔI at 1 Hz and 30 Hz by an average of 67.0% ($p < 0.001$) and 20.6% ($p < 0.05$), respectively ($n = 8$).

E. Frequency dependence of β -adrenoceptor mediated control of ΔI : The effects of 0.04 μM isoprenaline on the frequency dependence of ΔI represented practically a mirror image of that of NA (Fig. 3), although they were less marked. Isoprenaline changed the slope of

regression line in Fig. 3 to 0.8120 (control: 1.1013). The significance study based on repetitions of the type mentioned under B (Fig. 2) showed that this dose of isoprenaline increased ΔI at 1 Hz by 37.1% ($p < 0.001$, $n = 12$). Although the enhancing effect of isoprenaline on ΔI declined with a rise in frequency (Fig. 3), it did in part prevent the decay of ΔI per shock seen on stimulation at 30 Hz. Relatively to stimulation in the absence of isoprenaline, isoprenaline enhanced ΔI at this frequency by 26.5% ($p < 0.05$, $n = 12$). Thus isoprenaline differed from the other agents tested in having its maximal effects both at low and at high frequencies of stimulation (Fig. 5). Propranolol (1 μM) did not affect ΔI at any frequency (Fig. 3).

EFFECT OF DRUGS ON ^3H -NA SECRETION
HUMAN OMENTAL BLOOD VESSELS
FIELD STIMULATION 300 SHOCKS 120 V 0.3 ms

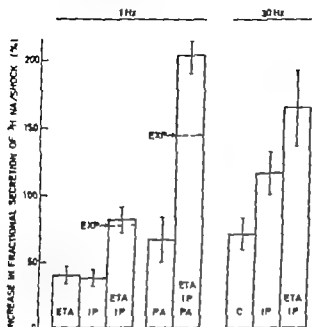


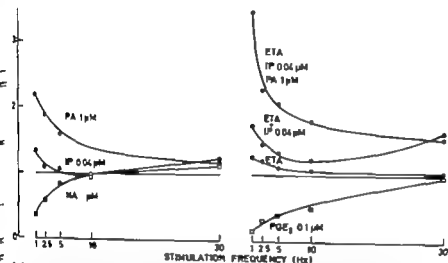
Fig. 2. Effect of drugs on Δt per shock stimulation for 2 periods (1 and 30 Hz) of Results. B. Overall results during control stimulation at 1 Hz \pm S.E. Asterisks indicate $p < 0.05$, $p < 0.001$. Abbreviations: IP = $0.04 \mu\text{M}$ PA = phentolamine $1 \mu\text{M}$. D. expected. Left panel: Drug-induced rise at 1 Hz. ETA and IP alone $p < 0.05$ controls. ETA+IP $p < 0.001$ in Δt or IP alone. PA $p < 0.001$ in Δt ETA+IP+PA $p < 0.001$ to PA alone. ETA+IP. Right panel: Stimulation at 30 Hz. Rise in Δt over control at 1 Hz. C = 30 Hz $p > 0.05$ to control at 1 Hz. IP $p < 0.05$ to control at 30 Hz. ETA+IP $p < 0.001$ to control at 30 Hz.

control stimulations, one at 1 Hz and one at 30 Hz, either with or without addition of different drugs (Fig. 2).

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FREQUENCY DEPENDENCE OF DRUG EFFECTS ON ^3H -NA SECRETION
 HUMAN ORIENTAL BLOOD VESSELS
 FIELD STIMULATION 300 SHOCKS 120 V 0.3 ms



5. Inverse relationship of the effect of drugs (exceptor: IP) to the frequency of stimulation. Ordinate at the presence of drug divided by Δt for control at the same frequency. Only means are shown. Left: Effects of adrenoceptor agonists and -antagonist. Right panel: Effects of PGE₂ and of ETA (to its formation of PGE₂). Effects of ETA + β -agonist or of ETA + β -agonist + α -antagonist. The figure shows only data from experiments with all frequencies 1–30 Hz (not those shown on Fig. 2).

At this frequency the additional effect of ETA treatment was a further rise in Δt by 22.4% ($p < 0.05$, $n = 23$). Because of this the shape of the curve describing the relative effect of α -isoprenaline on Δt (Fig. 5) resembles that describing the effect of isoprenaline alone, showing maximal effects at either low or high frequencies of stimulation, with a minimum stimulation at 10 Hz.

H. Effect of β -adrenoceptor stimulation after blocking α -adrenoceptors and PGE₂ formation. Addition of 0.04 μM isoprenaline in the presence of 1 μM phentolamine, after infusion of ETA, was found to cause a rise in Δt to the highest levels reached in the study on stimulation at 1–10 Hz (Fig. 3). As shown in Fig. 1 the fatigue effects were so marked that the actual slope of the regression line also shown in Fig. 3 becomes of doubtful significance (its average upward shift, when compared to the control line, was by 126%). As shown in Fig. 2 the combined effects of all three drugs on Δt at 1 Hz were more than additive (expected rise as the result of isoprenaline + phentolamine + ETA 144.5%, observed rise 207%). The effects of this combined treatment were highly significant, also when compared to the effects on Δt of ETA + isoprenaline (a further rise in Δt of 67.3%, $p < 0.001$, $n = 10$) and to those of phentolamine alone (a further rise in Δt of 82.9%, $p < 0.001$, $n = 8$). On stimulation at 30 Hz the combined treatment with isoprenaline + phentolamine + ETA showed an apparent rise in Δt per shock of 53.7% (about the same as with isoprenaline + ETA without phentolamine) when compared to the controls at this frequency ($p > 0.2$, due

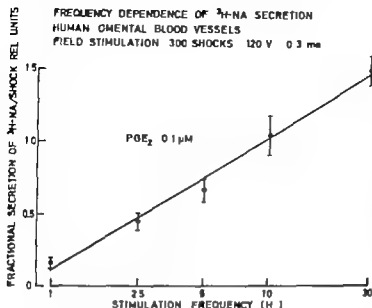


Fig. 4. Regression line of the frequency dependence during depression of the mechanism with PGE_2 . The line is semi-log plot where Δt was hyperbolic from the total duration of secretion per unit time. Mean Ordinate: Same relative to log Figs. 1-3.

F Frequency dependence of PGE_2 -mediated control of Δt Exogenous PGE_2 at 0 concentration depressed Δt on stimulation at all frequencies from 1 Hz to 10 Hz (at 30 Hz, see above under C and also Fig. 4). PGE_2 therefore depressed the regression describing the frequency dependence of Δt by 61.8%, while only moderately altering its slope (from 1.1013 to 0.8610). The effects of PGE_2 on Δt on stimulation at 1–10 Hz were significant ($p < 0.001$, $n = 4$). Relatively to control values at each frequency the effects of PGE_2 were inversely related to the frequency of stimulation (Fig. 5).

Infusion of ETA (60 μM concentration for 10 min followed by washing) to block formation of endogenous PGE_2 enhanced Δt significantly only on stimulation at 1 Hz. As shown in Fig. 3 infusion of ETA was found to cause a largely parallel upward shift of the regression line describing the frequency dependence of Δt by 15.0% without altering its slope (1.0034 as compared to 1.1013 for controls). In the experiments shown in Fig. 1 ETA was found to enhance Δt on stimulation at 1 Hz, by an average of 39.9% ($p < 0.001$, $n = 11$). Relatively to control values for Δt the effects of ETA were inversely related to the frequency of stimulation (Fig. 5).

G Effect of β -adrenoceptor stimulation after blocking PGE_2 formation The effects of 0.04 μM isoprenaline were very markedly enhanced after previous infusion of ETA to block formation of endogenous PGE_2 (Fig. 3). The regression line was shifted upward from the control level by an average of 39.4%, on stimulation at 1–10 Hz, and the slope was somewhat depressed (0.8821 against 1.1013 for the control). The results shown in Fig. 2 indicate that the effects of this combined treatment were purely additive, on stimulation at 1 Hz. While ETA or isoprenaline alone caused a rise in Δt by 39.9% and 33.5% respectively, the combined treatment was found to cause a rise in Δt of 81.5% (when compared to the effect of ETA alone, $p < 0.01$, $n = 11$). As expected from the enhancing effect of isoprenaline alone on Δt at 30 Hz the values for Δt at this frequency in the presence of isoprenaline and after ETA, were increased by 54.8% (Fig. 2) when compared to control at the same frequency ($p < 0.05$, $n = 11$). When compared to the effect of isoprenaline

nce of drugs strongly facilitating the secretory process (compare the moderate drift values during unfacilitated secretion, in the left panel of Fig. 1 with the very strong hysteresis during maximal facilitation, right panel). The accuracy of the Δt obtained is therefore inversely related to the degree of facilitation. In order to test statistical significance of the results separate experiments were carried out in which the stimulation was stimulated only twice (after the 2-3 initial control stimulations at 1 Hz), at lowest and highest frequencies used in the study (1 and 30 Hz), in the absence or presence of various drugs. The moderate scatter of data thus obtained at 1 Hz makes these data quite reliable, while the considerable scatter even with this approach makes it more difficult to correctly evaluate data at 30 Hz.

Basic frequency dependence of the secretory mechanism: In agreement with results obtained in many other tissues from several species (see Kirpekar 1975, Sjölrne 1975 b) it is found that the amount of neurotransmitter secreted per shock from the human motor nerves under study was highly dependent on the frequency of stimulation (cf Davies 1972). Also in agreement with results from other tissues, the frequency-dependent decline in the amount of transmitter secreted per shock, from the human vasomotor nerves, is to decline at frequencies higher than 10 Hz and particularly when drugs were added to facilitate NA secretion (Häefely *et al.* 1965, Kirpekar and Mitsu 1967, Davies and Longton 1968, Hedqvist and Sjölrne 1969, Sjölrne 1973). One possible explanation for the decrease in transmitter secretion per shock at 30 Hz is a decline in the size or number of vesicles reaching the terminals (Kirpekar 1975), on stimulation at this relatively high frequency. However the finding that Δt per shock in the present work became a simple proportional function of the total duration of depolarization per unit time, throughout a frequency range from 1 Hz to 30 Hz, when secretion was depressed (with PGE₂, Fig. 4), as it is likely that the fall in Δt per shock at 30 Hz, during normal or facilitated secretion, is due to failure in some link in the secretory mechanism, rather than to failure in conduction of nerve impulses. This is further supported by the similar findings of Kirpekar *et al.* (1975), that the tendency to a decline in NA secretion per shock at frequencies higher than 10 Hz, in phenylephrine-treated cat spleen, was largely prevented when the secretion was maintained at a low level by reducing the calcium concentration in the medium.

Frequency dependence of α -adrenoceptor mediated control of Δt : Both the effects of α -agonist NA and of the α -antagonist phentolamine were found to be inversely related to stimulation frequency. However there was a marked difference in their degree of frequency dependence, reflected in the effects on the slope of the regression lines shown in Fig. 3. The fact that the inhibitory effects of 1 μ M NA were apparent only at the lowest frequencies of stimulation need not imply that the secretory mechanism is not sensitive to the inhibitory effect of NA, on stimulation with frequencies of 10 Hz or higher. It could lead only to the conclusion that stimulation at the higher frequencies leads to accumulation of endogenous NA to concentrations higher than 1 μ M (neuronomuscular gap in human orontal muscle, 30-100-500 nM, Thureson-Klein *et al.* 1976, cf Bevan and Su 1974). Consistent with this is the finding that the enhancing effect on Δt of the competitive antagonist phentolamine declined more slowly with rise in stimulation frequency (Fig. 5), but ceased to be significant at 30 Hz.

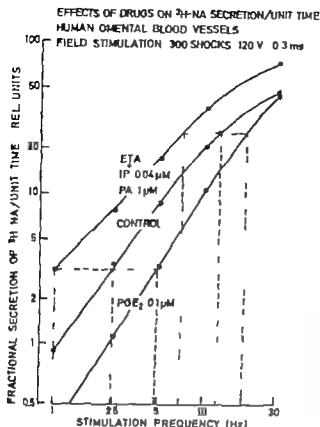


Fig. 6. Change in frequency required to balance facilitation (ETA + IP + PA) depression (PGE₂) of Δt per shock. For see the Text.

to the wide scatter shown in Fig. 1) The relative effect of the combined treatment isoprenaline phentolamine and ETA was therefore inversely related to the frequency of nerve stimulation (Fig. 5)

I Effects of drugs on the frequency dependence of Δt per unit time Since the decrease in neuro-effector transmission is the concentration of neurotransmitter at the level of postjunctional receptors, the values for Δt per unit time were calculated and plotted against the stimulation frequency (Fig. 6) comparing the control curve to those obtained during the highest degree of facilitation (ETA + isoprenaline + phentolamine), or inhibition (PGE₂) of the secretory mechanisms observed in the study. As shown in Fig. 6, a 5-fold rise in frequency would be required for the 'unfacilitated control transmitter secretion unit time to match that reached on stimulation at 1 Hz under maximal facilitation. PGE₂-mediated depression of the secretory mechanism a 5-fold rise in frequency from 1 to 5 Hz, would be required to reach the same level. At higher frequencies the difference decreases. To attain the value for Δt per unit time obtained on stimulation with facilitation with ETA + isoprenaline + phentolamine at a frequency of 7 Hz, the stimulation in the absence of facilitation would have to be raised by a factor of 1.81 Hz, and in the PGE₂-depressed state the frequency would have to be increased to 18.5 Hz.

Discussion

A Methodological difficulties. The main difficulty was the rapid onset of 'fatigue' of the secretory mechanism on serial stimulation at 1, 2.5, 5, 10 and 30 Hz. In particular

of drugs strongly facilitating the secretory process (compare the moderate drift along during unfacilitated secretion, in the left panel of Fig. 1 with the very big hysteresis during maximal facilitation, right panel). The accuracy of the Δt obtained is therefore inversely related to the degree of facilitation. In order to test statistical significance of the results separate experiments were carried out in which the neuron was stimulated only twice (after the 2-3 initial control stimulations at 1 Hz) at rest and highest frequencies used in the study (1 and 30 Hz), in the absence or presence of various drugs. The moderate scatter of data thus obtained at 1 Hz makes these quite reliable, while the considerable scatter even with this approach makes it more difficult to correctly evaluate data at 30 Hz.

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Frequency dependence of α -adrenoceptor mediated control of Δt : Both the effects of α -agonist NA and of the α -antagonist phentolamine were found to be inversely related to the frequency of stimulation. However there was a marked difference in their degree of frequency dependence, reflected in the effects on the slope of the regression lines shown in Fig. 3. The fact that the inhibitory effects of 1 μ M NA were apparent only at the lowest frequencies of stimulation need not imply that the secretory mechanism is not sensitive to the inhibitory effect of NA, on stimulation with frequencies of 10 Hz or higher. It could instead imply that stimulation at the higher frequencies leads to accumulation of endogenous NA to concentrations higher than 1 μ M (neuromuscular gap in human omental blood vessels: 30-100-500 nM, Thureson-Klein *et al.* 1976, cf. Bevan and Su 1974). Consistent with this is the finding that the enhancing effect on Δt of the competitive antagonist brexolamine declined more slowly with a rise in stimulation frequency (Fig. 5), but ceased to be significant at 30 Hz.

D Frequency dependence of β -adrenoceptor mediated control of Δt : The enhance of 0.04 μ M isoprenaline on Δt , on stimulation at 1 Hz, was only about 38%, as compared to 68–69% found in earlier studies of the same type of preparation (Stjärne and Brundin 1975 b, 1976 a). This difference may be due to the electrode arrangement and stimulation parameters used in the present study which also resulted in much higher control values of Δt , during the initial stimulation periods at 1 Hz (an average of $5.63 \cdot 10^{-4}$ s as compared to $2.45 \cdot 10^{-4}$ to $4.09 \cdot 10^{-4}$ s of the 3 H NA in the tissue at the time of stimulation, in the earlier studies). This may suggest that the effects of exogenous current and of blockade of β -adrenoceptors were in part directed against the same target in the secretory mechanism.

In agreement with earlier observations (Stjärne and Brundin 1975 b, 1976 a) phentolamine did not markedly affect Δt , at any stimulation frequency. This indicates that the secretory mechanism in these nerves was not to any important extent dependent on β -adrenoceptor mediated facilitation (Stjärne and Brundin 1975 b), as proposed by Adler-Gerendasy and Langer (1975) for various tissues.

Isoprenaline was unique in tending to enhance Δt both at low (1 Hz) and at high (30 Hz) frequencies. The effect at 30 Hz was not statistically highly significant (Fig. 2) together with the fact that ETA and isoprenaline in combination significantly enhanced Δt at 30 Hz (where ETA by itself was without effect) it indicates that β -adrenoceptor mediated facilitation sustained the secretory capacity on stimulation at higher frequencies.

E. Frequency dependence of PGE₂-mediated control of Δt : The extreme inhibition of exogenous PGE₂ previously demonstrated in human vasoconstrictor nerves on stimulation at 1 Hz (Stjärne and Gripe 1973; Stjärne and Brundin 1976 c) was verified to operate also at 2.5–10 Hz, although with decreasing efficiency at 30 Hz PGE₂ affected Δt . The same inverse relationship to stimulation frequency was observed for the enhancing effect on Δt of blockade of formation of endogenous PGE₂ with ETA. The effect of ETA on stimulation at 1 Hz in the present study was somewhat less than that observed with the electrode arrangement and stimulation parameters previously (39.9% as compared to 49.2% in the work of Stjärne and Brundin 1976 b).

F Interaction between the different control mechanisms: The combined effect of isoprenaline and ETA was additive, at least at 1 Hz (Fig. 2) and possibly reached its 'ceiling' at 30 Hz (Stjärne and Brundin 1975 b, 1976 b, work to be published). β -adrenoceptor mediated facilitation of Δt may thus be antagonized by endogenous PGE₂, although probably not by competition for the same target in the secretory mechanism (Stjärne and Brundin 1976 c).

The combined effect of ETA, isoprenaline and phentolamine was more than additive (Fig. 2). This indicates that the α -adrenoceptor mediated inhibitory effect of phentolamine on Δt is not or is only in part, directed towards the same target as that of the β -adrenoceptor mediated facilitation of the secretory mechanism. Work is in progress to analyze the interaction in more detail.

G Functional effects of interference with feedback control of Δt : The present results support those previously reported for several other tissues from various species concerning the complex local control of the amount of NA secreted from adrenergic terminals, of the efficiency of electro-secretory coupling and of propagated nerve impulses.

vasoconstrictor nerves of human omental blood vessels is clearly highly dependent on the frequency of nerve stimulation and on various local 'chemical' control mechanisms. One would expect that interference with this local control, and consequent increase in the amount of NA (number of NA quanta?) secreted per nerve impulse would lead to considerable change in the effective NA concentration at the level of the receptors of the effector tissue and therefore to alteration in neuroeffector transmission. The consequent alteration in neurogenic tone in blood vessels would lead to cardiovascular complications unless the altered situation is coped with by a reflexly mediated change in the firing frequency of the vasomotor nerves. The magnitude of the reflex adjustment of firing frequency which would be required *in vivo* to compensate for the altered release of NA per nerve impulse during the highest degree of depression or facilitation of secretory mechanism tested in the study is illustrated in Fig. 6. This estimation regards the fact that reuptake of transmitter was blocked in the present work. The plot indicates that the firing frequency in the nerves during depression of NA secretion by PGE₂ would have to be increased five-fold, from 1 Hz to 5 Hz, to maintain the NA output per time under facilitation by ETA/isoprenaline/pbentolamine.

One would therefore conclude that even though cardiovascular symptoms need not necessarily be apparent as a result of disturbance of mechanisms for local feedback control of NA secretion, since reflex changes in firing frequency could easily compensate alteration in the amount of NA secreted per shock, at least during resting conditions, it is quite possible that such symptoms may become manifest in situations where the cardiovascular system is exposed to increased strain.

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Effects of Sympathetic Nerve Stimulation in the Presence of a Slow Parasympathetic Secretion in the Parotid and Submaxillary Glands of the Rabbit

By

PER GJØRSTRUP

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Abstract

TITLE P *Effects of sympathetic nerve stimulation in the presence of a slow parasympathetic secretion in the parotid and submaxillary glands of the rabbit* Acta physiol. scand. 1977 101 211-218.

Salivary secretory responses from the parotid and the submaxillary glands of the rabbit were studied during sympathetic excitation, either alone or during slow parasympathetic background secretion, as the resting secretion normally present. The fairly rapid sympathetically evoked flow of saliva in parotid glands, as in the background secretion, further increased and obtained at low frequencies, at sympathetic stimulation above a low threshold. The effects, which could be repeated with cholinergic drugs in pilocarpine induced secretion, were isolated by α - and β -adrenoceptor blockade in combination. The submaxillary gland gave very scanty secretion on sympathetic excitation, tests being mediated via β -adrenoceptors. The responses were not increased in background secretion as their effect of sympathetic activation, as to retard the salivary flow. The retardation was attributed to vasoconstrictor accompanying sympathetic excitation. Eliminating the vasoconstrictor responses, retaining the sympathetic secretory effect, did not reveal any increase of the sympathetic salivary flow in the submaxillary gland.

Salivary secretion is generally considered to be controlled not only by parasympathetic, also by sympathetic nerves. There are striking differences, however, between the secretory responses to electrical excitation of the two types of nerves. Whereas the parasympathetic excitation regularly causes copious flow of saliva, which can be maintained over long time periods, the sympathetically evoked secretion is usually fairly slow even at high stimulation frequencies and tends to decline while stimulation is going on. The magnitude of the response varies between glands of different species and between different glands in the same species. There are even glands in which no salivary flow is obtained on sympathetic excitation. In the dog, for instance, no saliva is secreted from the parotid, but some from the submaxillary gland, although the acini of both glands are surrounded by adrenergic fibres (see Schneyer & Emmelin 1974).

In recent expts. on dogs and cats the sympathetic nerve was found to have a p secretory effect when stimulated during a slow parasympathetically evoked secretion the resting secretion normally present in the waking state (Emmelm and Gör 1976). The effect in the dog was particularly marked when sympathetic vasoconstriction prevented. Under these conditions a sympathetic secretory effect could be demonstrated in the parotid gland of this species.

In rabbits histochemical observations show that the acini both of submaxillary glands are well supplied with adrenergic fibres (Ehlinger, Garrett and Ohlin 19 and Engel 1970, Kagayama and Nishijama 1972, Garrett 1974, Bloom, Carlson and Son 1976). Physiological expts., on the other hand, indicate that sympathetic has a clear secretory effect in the parotid but very little or none at all in the submaxillary of this species (Nordenfelt and Ohlin 1957, Morley, Schachter and Smaje 1966, 1974, Smaje 1973, 1974). Nordenfelt and Ohlin (1957) also noticed that a slow secretion caused by pilocarpine could be accelerated by sympathetic stimulation when secretion was retarded, probably because of vasoconstriction.

In the present expts. the effects of sympathetic stimulation superimposed on a sympathetic background secretion were studied in the parotid and submaxillary of rabbit.

Methods

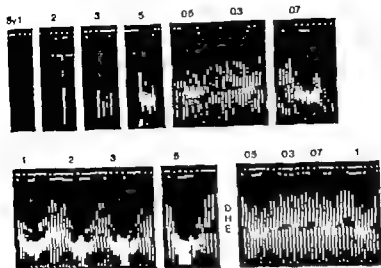
Rabbits of different strains, 3–4.2 kg, 39 females and 14 for the parotid gland. Urethane, 1.5 g/kg, was initially given through a lateral ear vein and femoral catheters additional doses of the anaesthetic and for injection of other drugs. A tracheal cannula was cannulated the submaxillary duct the conjugal tendon of the distal gastric muscle was divided gently freed from the surrounding tissues, from the distal aspect of the mylohyoid muscle down the middle of the gland. The preparation of the duct also made it possible to stimulate the chorda tendinea in its course along the duct. The parotid duct was exposed and cannulated as close to the mouth and the auriculo-temporal nerve was exposed as described by Burgen (1964) for cats and the sympathetic trunk was dissected. The neck. For measurement of blood flow through the submaxillary vein emptying into the external jugular vein except the maxillary gland for one were ligated between ligatures. Heparin 1 000 I.U./kg was given. A polythene tube of 1.7 mm diameter was inserted into the jugular vein, the other end being attached to a pharyngeal drop recorder.

Stimulation. A slow continuous flow of saliva, 1–3 drops/min, was produced by stimulating the tympanic membrane at a frequency of 0.5–3 Hz and the auriculo-temporal nerve at 0.7–2 Hz, using a drop recorder. The sympathetic was stimulated at frequencies from 0.07 to 10 Hz with a drop recorder and supramaximal voltage. Bipolar electrodes fitted with Grass stimulators, S4 and S44, stimulus isolation units were used. When pilocarpine was used to get a slow continuous flow of drug was infused by a syringe pump (Sage instrument model 375A) at a rate of 3 µg/kg min.

Recording. In the submaxillary gland the spontaneous secretion and the flow of saliva stimulated by the sympathetic alone were measured by connecting the cannulated horizontal duct to the other experimental glass canulae by drops of saline of 15 µl were used. Drop recorder made in the smoked drum. Drops of saliva were registered either by a standard water manometer or by an electromagnetic pen. Blood flow drops of blood were recorded by standard drop recorder. The blood pressure was measured by connecting the left femoral artery to a manometer.

Drugs. Pilocarpine, adrenaline, phenylephrine, isoprenaline, dihydroergotamine, phenol, procaine were given i.v.

Statistics. Student's *t*-test for paired observations was used. *P*-values less than 0.05 were considered significant.



Secretion from parotid gland. Records from above mention works, signal and secretion. In the first row the sympathetic (8y) frequencies in Hz as stimulated alone and in the other sections during slow parasympathetic secretion. The background secretion as evoked by stimulating the trigeminal nerve in the upper sections at 1 Hz, in the first 2 lower sections at 1.5 Hz and in the last at 3 Hz. Before the last section dihydroergotamine (DHE), 1 mg/kg, was injected.

Results

Parotid glands

One of the 14 parotid glands studied was there any sign of a spontaneous secretion. In 5 experiments the secretory responses to sympathetic stimulation alone were compared to those obtained when excitation occurred during a parasympathetic background secretion (as seen in Fig. 1) the sympathetic stimulated alone at 1 Hz did not give any secretion. 0.3 Hz was sufficient to accelerate the background secretion. The threshold frequency for sympathetic stimulation was lowered from 0.20 ± 0.10 to 0.15 ± 0.07 Hz (mean \pm S.E.), a difference which is highly significant ($P < 0.001$). Further the latency for the onset of sympathetic secretion was considerably shortened. The amount of saliva produced on sympathetic excitation was increased when stimulation was superimposed on the slow parasympathetic secretion: the average secretory responses were increased 1.3 Hz from $5 \mu\text{L/min}$ to $6.5 \mu\text{L/min}$. Maximal secretory responses were obtained around 3 Hz; higher frequencies inhibited the flow of saliva, probably because of the simultaneous activation of constrictor fibres in the gland.

The effect of sympathetic excitation, adrenaline or phenylephrine injections were also studied against slow secretion set up by infusion of pilocarpine (Fig. 2). The responses to sympathetic stimulation were very similar to those seen in parasympathetic secretion: the background secretion lowered the threshold from 1.83 ± 0.31 Hz to 0.13 ± 0.05 Hz, $n = 6$ ($P < 0.001$). Adrenaline also accelerated the pilocarpine elicited secretion and the threshold for adrenaline evoked secretion was lowered from $5.20 \pm 1.28 \mu\text{g/kg}$ to $0.07 \pm 0.03 \mu\text{g/kg}$, 5 ($P < 0.02$). Maximal responses were obtained with doses around $5 \mu\text{g/kg}$, while alone

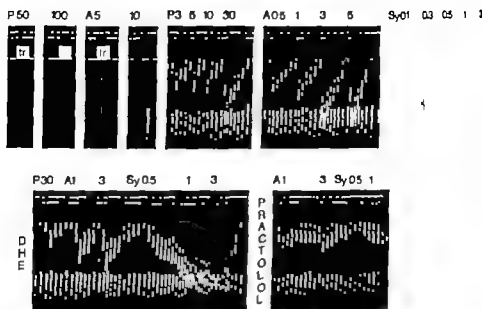


Fig. 2 Secretion from parotid gland. Record as in Fig. 1 (tr) traces of secretion. Secretion in 5 sections caused by drugs alone and in the others by drugs and sympathetic stimulation during pilocarpine in a dose of $3 \mu\text{g/kg/min}$. \square phenylephrine, A adrenaline. Doses in $\mu\text{g/kg}$ are given first, lower section dihydroergotamine (DHE), 1 mg/kg , was given and before the last section 1 mg/kg .

adrenaline in a dosage of 3 to $10 \mu\text{g/kg}$ in the different experiments only gave traces of saliva. In 3 expts. the α -adrenoceptor agonist phenylephrine in doses between $5 \mu\text{g/kg}$ barely evoked secretion, but in a pilocarpine secretion 1 to $3 \mu\text{g/kg}$ sufficed to the flow of saliva and maximal acceleration was obtained with 20 to $30 \mu\text{g/kg}$.

Blocking the α -adrenoceptors with dihydroergotamine 1–2 mg/kg , reduced the stimulating effects of sympathetic stimulation and of adrenaline (Fig. 1 ?), while the effects of phenylephrine were almost or completely abolished (Fig. 2). The remaining effects of superimposed sympathetic stimulation or of adrenaline administration however still produced at frequencies of stimulation or doses which acting as

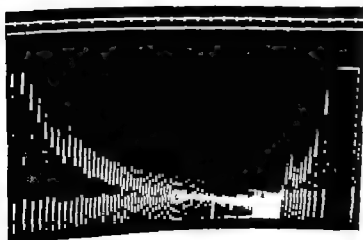


Fig. 3 Secretion from parotid gland. Records as in Fig. 1. Background secretion during infusion of pilocarpine $3 \mu\text{g/kg/min}$. This secretion continuously increased when maximal secretion was indicated by the signal (1). Detail section.

threshold for secretion. These responses were apparently not due to an incomplete block but to an activation of β -adrenoceptors, since they were greatly reduced by the β_1 -adrenoceptor blocking agent practolol, 2-4 mg/kg (Fig. 2). In two of four animals this agent in a dose of 2 mg/kg was seen to have a small transient accelerating effect on the background secretion. An intrinsic secretory property of this drug has also been observed in the rat (Ekstrom 1969).

Figure 3 shows a slow pilocarpine induced secretion that suddenly was accelerated and continued to be so for a period of 20 min until the animal gave up breathing. At the same time the pupils were widely dilated though the sympathetic trunk had been cut on the right side and 0.5 mg/kg of dihydroergotamine given 2 h earlier. The resemblance between this reaction and those on superimposed sympathetic stimulation or injections of adrenaline is likely that the increase in secretion was due to the release of catecholamines from terminals when the animal was in its final state. This idea is supported by the observed dilation of the pupil, which further indicates that the catecholamines were acting not on β - but also on α -adrenoceptors in this case.

Salivary glands

When the cannula was connected to the horizontal pipette a slow continuous flow of saliva, similar to the spontaneous, was seen (Nordenfliet and Ohlin 1957; Smajic 1973, 1974). It was found to be 0.25 μ l/min on the average ($n = 7$) and varying between 0.1 and 0.4 μ l/min in most animals when studied in 15 min periods.

Stimulation of the sympathetic trunk produced in most rabbits an additional 0.1-0.5 μ l of saliva at stimulation frequencies between 0.5 and 3 Hz; this additional secretion was not doubled at 5 Hz. The threshold frequency for secretion was in 1 animal found to be 1 Hz, in 5 animals 1 Hz, in 2 animals 2 Hz and in one 3 Hz. In 3 animals no secretion was noted on sympathetic excitation. Occasionally at 5 Hz and generally at higher frequencies a flow of saliva seen at the onset of stimulation successively declined during the stimulation period. When this was the picture the spontaneous secretion was slowed or even absent during the first minutes after stimulation. After administration of dihydroergotamine, 1-3 μ g, the tendency to decline was less marked and a more continuous flow of saliva was noted. The change after blockade was probably due to a reduced vasoconstriction. Blockade of the α -adrenoceptors did not, however, affect the amounts of saliva produced at frequencies between 0.5 and 3 Hz.

The saliva on sympathetic stimulation, whether secreted before or after α -adrenoceptor blockade, was always viscous. This necessitated interposed chorda stimulations in order to wash the sympathetic saliva otherwise causing obstruction. Further also seen in the experiments stimulation of the sympathetic did not give any increased flow; the first drops on the ongoing chorda stimulation were regularly more viscous than usual.

The effects of sympathetic stimulation when superimposed on a slow parasympathetically induced flow were as follows. In 8 rabbits no change in salivary flow was obtained, in 4 animals there was a small increase in salivary secretion, but judging from the experiments described above not greater than a mere addition of sympathetically evoked saliva. In a third group of 8 animals excitation of the sympathetic slowed the salivary flow rate. The

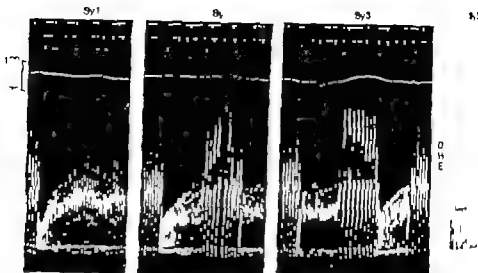


Fig. 4. Secretion and blood flow from submandibular gland. Records from above: minute mark signal, blood pressure and blood flow. Between signal mark the sympathetic is stimulated and parasympathetic secretion, indicated by 1, during the signal time. The chorda tympani is at 1 Hz. Before the last section dihydroergotamine (DHE) mg/kg i.v. is given.

retardation of the secretion was regularly accompanied by a retardation in blood flow from the gland (Fig. 4). To overcome the vasoconstriction, the most probable cause of decreased salivary flow, the α -adrenoceptor blocking drugs dihydroergotamine, 1 or phenoxybenzamine 5–10 mg/kg, were given. After α -adrenoceptor blockade stimulation frequencies were needed to retard the parasympathetic background and to reduce the blood flow through the gland. On lower frequencies the α -adrenoceptor blockade was not seen to have any marked effect on the secretory responses; the blood secretion was either unchanged, or only slightly increased although the vasoconstriction was eliminated (Fig. 4). In 8 rabbits injection of the β -adrenoceptor stimulating isoprenaline during a slow parasympathetic secretion regularly increased the salivary flow. With the dose given 0.1–5 μ g/kg, an additional 2–10 μ l of saliva was formed over a period of 2–4 minutes. When tested on its own isoprenaline, 5 μ g/kg gave a secretion about 5 μ l, except for one animal in which no secretion was seen. Secretory responses of the same magnitude to isoprenaline have earlier been reported by Ohlin (1964).

Discussion

In agreement with earlier reports the present experiments show that sympathetic stimulation causes a fairly rapid secretion from the parotid, but a minute response only or none at all from the submaxillary gland of the rabbit. The parotid secretion was mediated via α -adrenoceptors but not exclusively as pointed out by Nordenfält al (1957). Since the effect remaining after α -adrenoceptor block could be abolished by isoprenaline it can be concluded that it was evoked via receptors of the β -subgroup. Adrenoceptors of the β -type mediating salivary secretion in other species also belong to this subgroup (1972, Gjørstrup 1975).

The sympathetic secretory effect was greatly augmented in the parotid gland when

presence of slow parasympathetic resting secretion, an experimental situation con- to be more physiological than stimulating separately the sympathetic nerves (see and Emmelin 1961). The same was true when sympathetic nerve stimulation was d by sympathomimetic drugs. In these respects the gland reacts as salivary glands of d dogs (Emmelin and Gjorstrup 1975, 1976). In the dog submaxillary gland stimula- the sympathetic during a slow parasympathetic secretion produces an initial very low of saliva, followed by a phase of accelerated flow at a fairly well maintained level. a phase is attributed to myoepithelial contraction and the second to activity in se- cells (Emmelin and Gjorstrup 1974). However, the present increase in salivary flow be parotid gland occurred gradually and evenly, which makes it unlikely that there initial acceleration due to myoepithelial activity.

contrast, no augmentation could be detected in the submaxillary gland of the rabbit. gland the main effect of sympathetic stimulation was to reduce the background and a spontaneous secretion and in view of the pronounced vasoconstrictor effect ac- cpanying the stimulation, it is reasonable to attribute the retardation of the sali- ary flow diminished blood supply. Small secretory responses can be elicited by isoprenaline in bmaxillary gland of the rabbit, as also shown by Ohlin (1964). From this observation be inferred that the glandular cells have sparse supply with β -adrenoceptors and that oral sympathetic fibres may have access to some of these in most rabbits. The small ory effect of sympathetic stimulation is considered to be evoked via β -adrenoceptors e 1973, 1974). Hence, it is possible to study secretion when an α -blocking substance has ven to abolish vasoconstriction. Not even then could augmentation of the sympathetic be produced by supplying a background secretion.

the parotid gland of the dog adrenergic fibres are present in the acini (Fujiwara *et al* Garrett and Holmberg 1972), and a sympathetic secretory response, lacking when the ill excited alone, can be revealed by adding the background activity and abolishing sympathetic vasoconstriction (Emmelin and Gjorstrup 1976). The present analogous results do not support the view that sympathetic fibres greatly contribute to the trans- of water in the acini of the submaxillary gland of the rabbit. Recent work has shown submaxillary gland to receive less intimate and a less plentiful autonomic innervation, sympathetic and parasympathetic, than the parotid (Garrett, personal communication). may partly account for the difference between the glands as regards secretion of water sponses to sympathetic nerve stimulation. However, it cannot be the full explanation. As seen in methods, the secretory responses to parasympathetic stimulation are similar th glands.

It is reasonable to attribute other functions to the adrenergic nerves surrounding the acini e submaxillary gland. They may be of importance for the secretion of proteins: this is ported by the present finding that the submaxillary saliva became erythrocytic when the pathetic nerves were activated. Langley already in 1879 pointed out that both the parotid the submaxillary glands became degranulated on sympathetic excitation. Although not ported by the present experiments, it is also conceivable that the sympathetic acinar nerves : part in the control of the myoepithelial cells surrounding the acini, as is the case in dogs.

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Fever and Behavioural Temperature Regulation in the Frog *Rana esculenta*

By

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Abstract

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Ant and colonic temperatures were recorded in frogs (*Rana esculenta*) which had selected suitable environments in a box filled with 2.5 cm water. The skin temperatures ranged from 0°C to +40°C. Measurements were performed before and after intraperitoneal injections of killed pathogenic bacteria (*Aeromonas* and *M. mace*), killed non-pathogenic bacteria (*M. mace* II) and 0.5 µl sterile saline, intramuscular injections of blood plasma from frogs pre-injected with killed *M. mace* or injections of PGE₁ in vivo. The injections of pathogenic bacterial endotoxins caused, after latencies of 5-120 min, higher pre-4 star temperatures. Each produced an average maximum colonic temperature increase of 6.5°C + (S.E.) (p < 0.001). The non-pathogenic bacteria and sterile saline caused no temperature change. Systemic hyperthermia of shorter latency, as caused by injections of blood plasma from frogs pre-injected with *M. mace*. Monophasic hyperthermia of the shortest latency was observed after intraperitoneal injections of PGE₁. Based on their similarity we suggest that ectothermic and endothermic fever have a phylogenetic origin.

Key words: Frog, behaviour, temperature regulation, fever, prostaglandins.

It is a pathological condition with raised core temperature maintained by activation of thermoregulatory mechanisms of the organism. This manifestation of disease is characteristic for most mammals during inflammations and has been extensively studied and reviewed (Bligh 1973, Hensel 1973, Hellon 1974).

Both bacteria and viruses produce fever by injection into mammals (Hellon 1974). This fever is characterized by a typical pattern of temperature changes including a dose-response relationship concerning maximum temperature increase, latency and duration of the fever. It is generally accepted that the exogenous pyrogens induce release of endogenous pyrogens in the organism (Bligh 1973, Hellon 1974). Further several authors (Cooper, Cranston and O'Connor 1967, M. Lion and Wendlandt 1970, Feldberg and Gupta 1973, Stitt 1973, Cooper

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Preston and Veal, 1976) have produced evidence suggesting that the central action of endogenous pyrogens consists of initiating synthesis of Prostaglandin E (PGE_1) which modifies the thermoregulatory neuronal activity in the CNS.

Recently Vaughn, Bernheim and Kluger (1974) have reported that when the lizard *Dipsosaurus dorsalis* was injected with killed Gram-negative bacteria (*Aeromonas* in it selected higher ambient temperatures and increased its cloacal temperature by approximately 2°C. Reynolds, Castorlin and Covert (1976) injected endotoxin from the same bacteria into two species of fish (*Lepomis macrochirus* Rafinesque and *Allopieternis salmoides*) and observed that they increased their average preferred water temperature by 2.6°C. When killed *Aeromonas hydrophila* was injected into tadpoles of *Rana catesbeiana* and *Rana pipiens* they selected 2.6°C and 2.7°C higher water temperatures (Castorlin and Reynolds, 1977).

The role of behaviour in producing fever in neonatal endotherms was demonstrated by Satinoff, McEwen, Jr and Williams (1976). Newborn rabbits maintained under isothermal conditions did not develop fever upon injection of exogenous pyrogen, but when placed in a thermal gradient they increased their rectal temperature with 0.9°C to a warmer location.

The role of mammalian fever in decreasing the mortality during bacterial infection is disputed (Bennett and Nicastri 1980). However, in the lizard *Dipsosaurus dorsalis*, the survival value of the behavioural fever response has been indicated (Kluger, Rungler and Amel, 1976; Bernheim and Kluger 1976a). After receiving injections of live *Aeromonas hydrophila* tadpoles were allowed to increase their temperature behaviourally, maintained at their preferred temperature. Tadpoles injected with sodium salicylate which depressed the fever response had the highest per cent of survival was observed in the first group. Comparative studies of ectothermic and endothermic fever might shed light on the concept of a common phylogenetic origin and thus the role of fever as a possible defense mechanism.

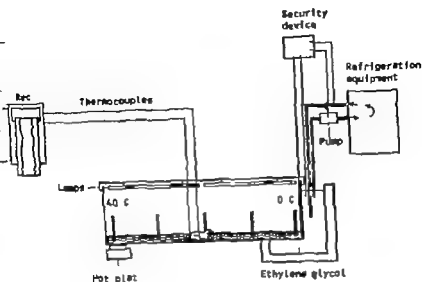
The frog *Rana esculenta* selects a location in a thermal water gradient which provides cloacal temperatures between 25°C and 28°C (Cabanac and Jeddi 1971). This temperature-regulating behaviour is triggered by signals from spinal thermoreceptors (Ducloir, Farrel and Cabanac 1973). It thus represents a useful preparation for studying the characteristics of behavioural fever after injections of pyrogen.

In this study the thermoregulatory behaviour of the frogs was studied after intraperitoneal injections of killed pathogenic and nonpathogenic bacteria. Further, another group of frogs received intraperitoneal injections of blood plasma from frogs pre-injected with pathogenic bacterial endotoxin, while a third group received injections of PGE_1 into the brain.

Materials and Methods

Animals. Frogs (*Rana esculenta*) weighing from 15 g to 1.5 g were bought at the local market and without food at room temperature. Only healthy frogs, as judged by their behaviour, were used.

Temperature gradient. The temperature gradient was established in a box made of galvanized steel, 15 cm long, 15 cm wide, 37 cm deep (Fig. 1), filled with 3 cm of water. The water temperature in the middle of the box was maintained at 10°C by circulating cooled ethylene glycol. Surrounding insulated cement. An adjustable heater was located underneath the other end of the box and maintained the water temperature in this end at about 40°C. The thermoregulatory behaviour of the frogs was studied



Schematic drawing of the experimental design modified from Declercq, Festens and Cabiaux

To reduce possible disturbance of the frog from the surroundings the gradient was uniformly illuminated by overhead lamps and partly covered by lead. Observation of the animal was accomplished by projection of mirrors.

Temperature measurements. Temperatures were measured by 36-gauge nylon coated copper-constantan couples with the reference junctions in melting ice, and registered on Polycomp 2, 12 channel recorder (Harrison & Brown A.G.). The accuracy of the measurements was $\pm 0.3^\circ\text{C}$.

Water temperature (which was preferred by the animal) was obtained by entering thermocouple to 1 cm in the abductor.

Air column temperature as measured by thermocouple 3 cm into the column, tested by external air.

Thermocouple leads did not disturb the frog's movement in the gradient.

Experimental procedure. The general protocol was to allow the frogs to select preferred temperature thermal gradient before and after injections of pyrogens. Group 1 received intraperitoneal injections of bacterial endotoxin, group 2 blood plasma from frogs pre-injected with bacterial endotoxin, while group 3 received injections of Prostaglandin E_2 into the brain. Skin and column temperatures were continuously noted during the whole experiment.

Frogs were anesthetized by equipping the frogs with the thermocouples. In order to diminish the usual reflex shivering responses as produced by cooling the frog in ice. The animal was then put into the gradient, reached equilibrium, and left undisturbed until it had selected preferred water temperature (± 1 at least 30 min in the water location). The frog was then carefully removed from the gradient and oxygen was administered. Following the injection the frog was replaced in the gradient at its preferred ice, and left undisturbed throughout the experiment. Care was taken to avoid noise and any disturbance of spontaneous animal.

Parental pyrogen. Ten frogs were injected with bacterial pyrogen. Three strains of *Mycobacterium* were used: *M. paratuberculosis* and *M. avium* H7 were used. The bacteria were killed by heat (80°C), suspended in

0.9% saline (1 mg/ml) and stored in the refrigerator (-4°C). The frog received 2 ml of this solution. Each frog also received as a control injection of 0.1 ml sterile 0.9% saline. The control experiments and final experiments were performed at the same time of the day.

Disposable, sterile needles and syringes were used for all injections. The solutions of killed *Mycobacterium* were transferred to the syringe close to the flame from Bunsen burner in order to avoid contamination of solutions by foreign bacteria. Before injection all solutions were brought to room temperature.

Preston and Veale (1976) have produced evidence suggesting that the central action of endogenous pyrogens consists of initiating synthesis of Prostaglandin E_2 (PGE₂) which modifies the thermoregulatory neuronal activity in the CNS.

Recently Vaughn, Bernheim and Kluger (1974) have reported that when the lizard *Sauropterus dorsalis* was injected with killed Gram-negative bacteria (*Aeromonas hydrophila*) it selected higher ambient temperatures and increased its cloacal temperature by approximately 2°C. Reynolds, Castelin and Covert (1976) injected endotoxin from the bacteria into two species of fish (*Lepomis macrochirus* Rafinesque and *Micropterus salmoides*) and observed that they increased their average preferred water temperature by 2.6°C. When killed *Aeromonas hydrophila* was injected into tadpoles of *Rana catesbeiana* and *R. pipiens* they selected 2.6°C and 2.7°C higher water temperatures (Castelin and Reynolds, 1977).

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The role of mammalian fever in decreasing the mortality during bacterial infection is disputed (Bennett and Nicastri 1950). However, in the lizard *Dipsosaurus dorsalis*, the survival value of the behavioural fever response has been indicated (Kluger, Ringler and Auerbach, Bernheim and Kluger 1976a). After receiving injections of live *Aeromonas hydrophila* were 1) allowed to increase their temperature behaviourally, 2) maintained at their normal temperature, 3) injected with sodium salicylate which depressed the fever response. The highest per cent of survival was observed in the first group. Comparative studies of ectothermic and endothermic fever might shed light on the concept of a common phylogenetic origin and thus the role of fever as a possible defense mechanism.

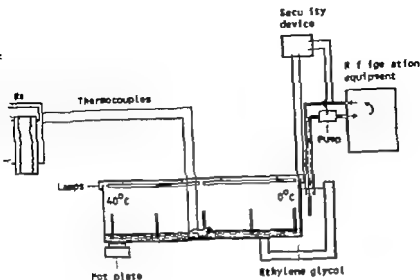
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In this study the thermoregulatory behaviour of the frogs was studied after intraperitoneal injections of killed pathogenic and nonpathogenic bacteria. Further, another group of frogs received intraperitoneal injections of blood plasma from frogs pre-injected with pathogenic bacterial endotoxin, while a third group received injections of PGE₂ into the brain.

Materials and Methods

Animals. Frogs (*Rana esculenta*) weighing from 15 g to 125 g were bought at the local market and kept without food at room temperature. Only healthy frogs, as judged by their behaviour, were used.

Temperature gradient. The temperature gradient was established in a box made of galvanized iron, 15 cm high, 15 cm wide, 37 cm deep (Fig. 1), filled with 3 cm of water. The water temperature in the box was maintained at 0°C by circulating cooled ethylene glycol. A surrounding insulated compartment. An adjustable heater was located underneath the other end of the box and maintained the temperature of this end at about 40°C. Fifteen thermometers were spaced at regular intervals along



Schematic drawing of the experimental design (modified from Daddar, Faurio and Cabaret)

reduce possible disturbance of the frog from the surroundings (the gradient is uniformly fixed by overhead lamps and partly covered by lid). Observation of the animal is accomplished by means of mirrors.

General measurements. Temperatures were measured by 36-gauge nylon coated copper-constantan couples, with the reference junctions in melting ice, and recorded on Polycorp 2, 12 channel bench recorder (Hartman & Braun A 13). The accuracy of the measurements was $\pm 0.3^\circ\text{C}$.

Water temperature such as preferred by the animal is obtained by inserting thermocouple to an area on the abductor.

Colonic temperature is measured by thermocouple 3 cm into the colon, fastened by external pin.

Thermocouple leads did not impede the frog's movement in the gradient.

General procedure. The general protocol is to allow the frogs to select preferred temperature thermal gradient before and after injections of pyrogen. Group 1 received subperitoneal injections of bacterial endotoxin, group 2 blood plasma from frogs pre-injected with bacterial endotoxin, while group 3 received injections of *Proteoglycan E₁* into the brain. Skin and colonic temperatures were continuously recorded during the whole experiment.

Experiments are initiated by equipping the frogs with the thermocouples in order to diminish the animal's reaction to the anesthesia as produced by cooling the frog in ice. The animal is then put into the gradient between temperatures, and left undisturbed until it had selected preferred water temperature (1-2 h at least 30 min in the same location). The frog is then carefully removed from the gradient and injection is administered. Following the injection the frog was replaced in the gradient at its preferred temperature, and left undisturbed throughout the experiment. Care is taken to avoid noise and any disturbance of experimental animal.

Animal pyrogen. Ten frogs are injected with bacterial pyrogen. Three strains of *Mycobacterium avium*, *M. farci* and *M. neoaurum* are used. The bacteria were killed by heat (80°C), suspended in 0.9% saline (1 mg/ml) and stored in the refrigerator (-4°C). The frog received 2 ml of this solution. Each frog also received an intraperitoneal control injection of 2 ml sterile 0.9% saline. The control experiments and trials were performed at the same time of the day. Disposable, sterile needles and syringes were used for all injections. The solutions of killed *Mycobacterium* transferred to the syringe close to the flame from Bunsen burner in order to avoid contamination by foreign bacteria. Before injections all solutions were brought to room temperature.

Transfer of endogenous pyrogen Killed *A. ranarum* was i.p. injected into 5 donor frogs in doses ranging from 53 μ g to 75 μ g. About 2 h later the blood was drawn and the frog killed.

The blood sampling procedure consisted of carefully dissecting the heart free and inserting a fine needle into the ventricle. After injecting a small amount of heparin the blood was drawn into a syringe and centrifuged for 10 min at 3 000 r.p.m.

0.5 to 2.5 ml of this plasma was i.p. injected into 4 recipient frogs according to the general procedure in the experiments.

Prostaglandin E The prostaglandin was supplied as PGE₁. It was made soluble in 0.9% saline by converting it to PGE₁-Na as described by Skitt (1973). Stock solutions of 10 mg PGE₁-Na/ml and 1 mg/ml were made. All glassware used in this connection was scrupulously cleaned and dried.

5 frogs received PGE₁ injections. After they were removed from their initial stay in the gradient they were locally cooled by ice cubes. The skull was exposed and a hole carefully made with a drill slightly left of the midline, in an area corresponding to the diencephalon.

The injections into the brain were administered by a Hamilton syringe fastened in a micro-manipulator. The syringe needle was carefully lowered down through the hole until its free progress was arrested by bony structures of the palate. The needle was then retracted 1 mm and the solution injected.

In every experiment 0.5 μ l of PGE₁-Na solution was injected. The control injections were done later in the day and consisted of 2.5 μ l of the saline vehicle, administered through the same hole in the skull.

When the experiment was terminated, 0.5 μ l of 5% methylene blue was injected according to the dissection procedure, and the animal killed by decapitation. The brain was dissected free, fixed in a 10% formalin solution and macroscopically inspected to verify the site of the injection.

Treatment of the results

The statistical significance of the observed response was assessed with Student's *t*-test.

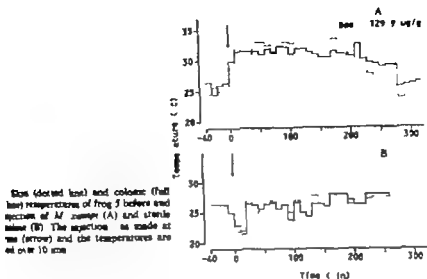
Results

Bacterial pyrogen

A Typical response Fig. 2 shows the results from 2 experiments on one frog (5), which is representative for the study. The animal settled down with a skin temperature of about 26°C and a colonic temperature of 26°C. After receiving an i.p. injection of *Mycobacterium xenopi* (panel A) the frog moved to warmer water and during 220 min the skin temperature varied between 30.8°C and 33.3°C while its colonic temperature was maintained between 30.5°C and 32.5°C. After this period of fever the frog returned to cooler water, indicated by the rapidly falling skin temperature, followed by a slower drop of the colonic temperature. When the frog, on the previous day, received an i.p. injection of sterile saline (Fig. 2, panel B), no major change of location occurred except for an initial 'cool trip'. The frog's colonic temperature was most of the time maintained between 24°C and 27°C, increasing to 28°C in the last hour.

As a general trend, illustrated in Fig. 2, the skin temperature remained higher than the colonic temperature during the fever period but lower than the colonic temperature when the frog returned to its pre-injection thermal condition. In the control experiments the skin temperature oscillated around the colonic temperature. Further, when the frog was removed from its selected location in the gradient, it always returned quickly. This observation is valid whether the animal was hyperthermic or normothermic.

B Average response Fig. 3 panel A shows the mean colonic temperatures of 7 frogs (3, 4, 5, 7 and 8) before and after the injection of *Mycobacterium xenopi* in doses ranging from 16.1 μ g to 129.9 μ g. The average response of the 7 frogs is not calculated before 150 min after the injection since the first frog terminated its fever at this time.



Skin (dotted line) and colonic (full line) temperatures of frog 5 before and after injection of *M. xenopi* (A) and sterile saline (B). The injection is made at time (arrow) and the temperatures are read over 10 min.

and that, following the injection, the frogs increased their colonic temperatures, *Le* the feverish.

In the control expts., shown in the same panel, all of the frogs except one were injected with sterile 0.9% saline (frog 2 died after being accidentally affected by the needle in the 1 cord). The control injections produced no hyperthermia. A slight elevation of the skin temperatures towards the end of the expts. was, however, noted (Fig. 3 A and 2 B). To investigate if this observation was due to diurnal temperature pattern, all colonic temperatures obtained in the control expts. were related to the time of the day. A regression line was fitted according to the method of the least squares. Although this indicated a 1°C higher colonic temperature at 5 o'clock p.m. compared to at 11 o'clock a.m. this trend was statistically non-significant ($p > 0.5$).

Two frogs (10, 11) were injected with *Myxobolus ranae* in doses of 47.6 µg/g (10) and 129.9 µg/g (11). Within 40 min frog 11 had moved to warmer water and increased his colonic temperature from 22.8°C to 31.9°C. It then died during the hyperthermia, 60 min after the injection. Examination of the body did not reveal any signs of internal damage caused by the parasite. Frog 10 exhibited latency of 20 min and then entered a hyperthermic period lasting 120 min. The fever described a clear biphasic pattern. The control injection of sterile 0.9% saline produced no hyperthermic response (Fig. 3 B).

The maximum colonic temperature increase, maintained for 30 min, averaged from the frogs which received injections of *M. xenopi* and *M. ranae* was $6.5^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ (S.E.M.) ($p < 0.001$), while the corresponding temperature change at the same time after injecting sterile saline was non-significant ($p > 0.3$).

Non-pathogenic bacterium When 2 frogs (5, 9) were injected with *Myxobolus ranae* in doses of 139.9 µg/g and 56.3 µg/g respectively no hyperthermic response was observed (Fig. 3 C).

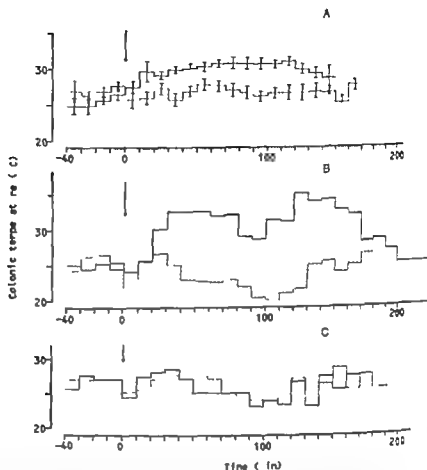


Fig. 3. Panel A shows the average colonic temperatures and standard error of the mean (vertical bars) before and after injection of killed *A. aeropyrum* concentrations ranging from 16.1 µg/g to 134 µg/g (drawn line) in frogs 1, 2, 3, 4, 5, 7 and 8, and sterile 0.9% saline (dotted line) in frogs 1, 3, 4, 5, 7. Panel B exhibits colonic temperatures in one frog (10) injected with 47.6 µg/g killed *A. aeropyrum* (drawn line), and sterile 0.9% saline (dotted line). Panel C shows the colonic temperatures in two frogs (1 and 9) injected with 36.3 µg/g killed *A. aeropyrum* (drawn line) and sterile 0.9% saline (dotted line). Further as in Fig. 1.

D Characteristics of the response. The maximum colonic temperature increase obtained for 30 min, during fever in each frog showed a tendency to increase up to the 60 µg/g, while at the highest dose (130 µg/g) a lowered response was observed (Fig. 1).

Latency is in this study defined as the time elapsed between the injection and the onset of colonic temperature increase. As illustrated in Fig. 4 B latencies of 120 min and 5 min were observed at the lowest and highest dose of bacteria respectively while those of the intermediate doses ranged from 10 min to 40 min.

Two frogs were still hyperthermic when the experiments were terminated after fever durations of 170 min and 300 min. The other frogs returned to cooler water exhibiting fever durations ranging from 90 min to 740 min (Fig. 4 C).

Transfer of endogenous pyrogen

Table I shows the characteristics of the response observed in four frogs (20) injected with blood plasma from frogs pre-injected with *A. aeropyrum* after a la

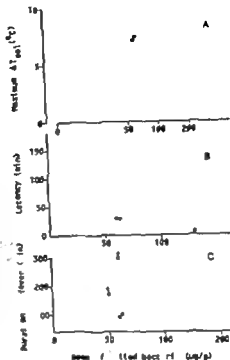


Figure 1. A shows the maximum increase of the temperature which was sustained for 30 min in the logarithm of the administered dose of bacteria. (Panel B and C the latency and duration of the hyperthermic response are related to the dose of killed bacteria given. Closed circles, *per M* concept; open circles, injections of *1/10* the small arrows in panel C indicate that the frogs still hyperthermic 30 min after the injection.

frogs moved towards warmer water and displayed a monophasic hyperthermia of moderate size, lasting 16–20 min. The mean increase of the colonic temperatures ranged from 1.5 to 5.3 $^{\circ}C$. Two of the frogs died respectively 30 min and 3 h after the injection, but the cause of the deaths could not be determined. When 3 frogs received control injections of 1:1 plasma from normal frogs, no change of colonic temperatures occurred.

Experiment E₂

1 μ l of the Prostaglandin E₂ solution was injected into the diencephalon of 5 frogs (9, 15, 16, 18) in doses ranging from 54 ng/g to 141 ng/g. The response (Table II) followed a general

TABLE II. Characteristics of the hyperthermic responses elicited by the frogs receiving intraperitoneal injections of blood plasma from frogs pre-injected with killed *M. ranas*.

S	Injection		Latency of response (min)	Duration of response (min)	Mean increase of $T_{colonic}$ ($^{\circ}C$)
	Amount (ml)	Dose (μ l/g)			
9	2.5	65.6	4	20	3.9
15	0.5	76.5	4	20	3.0
16	0.5	18.3	4	16	2.0
18	2.0	100.0	3	20	3.3

Dead 30 min after the injection.
Dead 3 h after the injection.

TABLE II Characteristics of the hyperthermic responses exhibited by the frogs receiving prostaglandin E_1 (PGE_1) into the brain. The bracketed numbers denote the colonic temperature deviations from the pre-injection levels, obtained after the control injections at the time corresponding to the PGE_1 induced hyperthermia.

Frog	PGE		Latency of response min	Duration of response min	Mean increase of T colonic °C
	Amount μ g	Dose ng/g			
9	5	141		65	5.8
15	2.5	69		35	8.8 (-0.8)
16	2.5	58	2	25	9.7 (1.0)
16	2.5	58		20 ^a	6.5
17	2.5	54	3	28 ^a	7.5

The animals died during the hyperthermic phase

pattern which is illustrated by one expt. (frog 15) in Fig. 5. After a latency of 2 min it moved towards warmer water thereby producing a monophasic hyperthermia lasting 1 min. Control injection in the same brain area produced no hyperthermic response. Frogs (16, 17) developed signs of nervous disorder (impaired and jerky movements of its extremities) shortly after the injections. They did, however move to warmer locations and died there 22 min (frog 16) and 31 min (frogs 17) after the injection (Table II). A possible explanation could be that with these two particular frogs physical damage to the ventral thalamus was caused by the syringe needle. Since the ventral thalamus is a motor coordinating centre (Romer 1970 p. 519) in the frog, the observed motoric dysfunction could well result.

Macroscopic examination of the brains showed that the frogs were injected in the diencephalon slightly left of the midline and close to the optic chiasma.

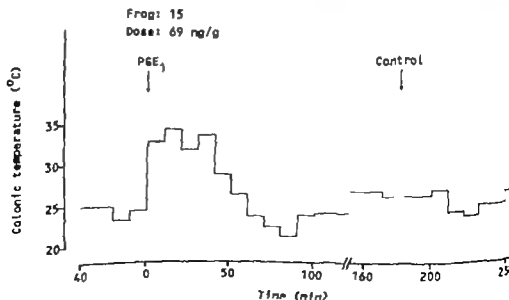


Fig. 5 The colonic temperatures of one frog (15) before and after injection of 2.5 μ g Prostaglandin (PGE_1) and sterile 0.9% saline (control) into the diencephalon. The Prostaglandin solution was injected at zero time and the saline 180 min later.

Discussion

ogs in the present study voluntarily selected water temperatures which produced temperatures between 25°C and 28°C thus confirming the observations of Cabanac (1971) and Duclaux, Fantino and Cabanac (1973). When killed *M. xanthi* and *M. m* were injected i.p. in doses ranging from 16 µg/g to 130 µg/g, the frogs shifted their to warmer water which significantly increased their colonic temperatures ($p < 0.001$). *M. xanthi* is pathogenic towards anurans (Schwabacher 1959), *M. m* is clearly thogenic and *M. m* is only suspected pathogenic (Carré 1973). The fever re- produced by the frogs which received endotoxins from *M. m* may lend support possible pathogenicity of this bacterium, since no fever response was encountered when *M. m* was injected. These observations show that a behavioural fever was produced p. injections of endotoxins from pathogenic bacteria. Thus it is evident that in addition rring i reptiles after injection of live and dead *Aeromonas hydrophila* (Vaughn, m and Kluger 1974; Bernheim and Kluger 1976 a, b), fish (Reynolds, Casterlin and 1976) and amphibian larvae (Casterlin and Reynolds 1977), behavioural fever occurs adult amphibian.

dowing injections of exogenous pyrogen the hyperthermia occurred after delays from to 120 min. When recipient frogs were injected with blood plasma from frogs pre d with *M. m* all observed latencies were shorter in accordance with Grant and ns (1953) observations in rabbits. Although the present results are too few to rep- t conclusive evidence, they may indicate that the blood of the donor frogs contained ene substances different from bacterial endotoxins since the latencies were shorter o shortest latency was observed when PGE₁ was injected into the hypothalamic region frog's brain. This is in accordance with Sitt's (1973) observations in rabbits and indi- hat PGE₁ in this brain region of the frog induce a hyperthermic response brought about lecting higher preferred temperature.

latency observed when exogenous pyrogen was i.p. injected could be due to delayed e absorption. However the consecutive shortening of the latencies in the three groups ps may also indicate that the exogenous pyrogen did not exhibit its effect directly on but acted through release of endogenous pyrogen which in its turn modified the il nervous thermoregulatory activity in connection with synthesis of PGE. This is a ally accepted view of the processes involved in generating fever in mammals (Helson).

duration of the frog's fever response and the relationship between the colonic tem- ture bacterium and the dose of bacterial endotoxin were similar to corresponding observa- in rabbits (Eichenberger *et al.* 1966, Lechat et Delcru 1964), supporting the indicated larity between ectothermic and endothermic fever.

fever obtained in rabbits after injecting pyrogenic blood was similar to bacterial ed fever regarding magnitude and duration (Grant and Whalen 1953). When the frogs njected with blood plasma from donor frogs pre-injected with *M. m* the magnitude duration was modest compared to when exogenous pyrogen was injected. This may be o several factors. Observations of Grant and Whalen (1953) and Snell (1961) suggest low

concentrations of endogenous pyrogen in the blood of rabbit and man, and we obtained volumes of blood from the donor frogs. Finally according to Grant and Whalen (1959) pyrogenicity of the rabbit's blood was dependent upon the time elapsed after bacterial toxin was injected. Accordingly we may not have drawn the blood of the frog at its maximum pyrogenity.

When PGE_1 was injected into the hypothalamus of the frog the magnitude of the colonic temperature increase was in the range observed when bacterial endotoxin was injected. This pattern is in accordance with results obtained in rabbits (Stitt 1973, Calhoun, Preston and Veale 1976) and represent a further support of the similarity in the pattern of generating ectothermic and endothermic fever.

Finally the present study permits some conclusions about the organization of temperature regulation in amphibians. The existence of cutaneous temperature sensitivity is important since the frogs orient themselves in a thermal gradient (Cabanac and Jedou 1971, Doolittle, Fantino and Cabanac 1973). When the frogs in the present study were moved away from their thermopreferendum they promptly returned before any change of the colonic temperature was evident. This observation further supports the existence of skin thermoreceptors. External signals from spinal temperature receptors have been demonstrated (Ducourt, Fantino and Cabanac 1973) and the fever induced by PGE_1 injections in the hypothalamus of the frog suggests that this brain region is involved in temperature regulation.

When the frogs in the present study had selected a preferred water temperature, the external signals in their thermoregulatory system presumably equalled some reference level (set point). The selection of a higher thermopreferendum by the frogs after injections of pyrogenic substances is most easily explained by a raised set point, which at pre-injection level of preferred water temperature will induce heat seeking activities. This observation is in connection with the challenge of the concept of set point in temperature regulation (Schell, Snelken and Atkins 1970, Houdas *et al.* 1973).

d Alecy and Kluger (1975) and Pittman *et al.* (1976) have produced evidence supporting a similarity between avian and mammalian fever. Although endothermic fever is the result of increased metabolism and peripheral vasoconstriction in addition to behaviour while ectothermic fever is a sole behavioural response, the present results may indicate that the responses are initiated through similar intermediary steps. Since the general pattern of temperature regulation in amphibians is similar to that in mammals, the present results support the theory of a common phylogenetic origin of ectothermic and endothermic fever.

The present work was supported by l'Institut National de la Santé et de la Recherche Médicale (I.N.S.E.R.M.), le Centre National de la Recherche Scientifique (C.N.R.S.), la Fondation pour la Recherche Médicale Française and The Norwegian Research Council for Science and the Humanities. We are indebted to Professor J. Vallier-Raynard, Department of Bacteriology and Doctor J. E. Pike, The Upjohn Company, who provided free bacteria and prostaglandin respectively.

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The present work was supported by l'Institut National de la Santé et de la Recherche Médicale (I.N.S.E.R.M.), le Centre National de la Recherche Scientifique (C.N.R.S.), la Fondation pour la Recherche Médicale Française and The Norwegian Research Council for Science and the Humanities. We thank Professor J. Vallier Raynard, Department of Bacteriology and Doctor J. E. Pike, The University of Colorado, who provided free bacteria and prostaglandin respectively.

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Recovery is to a large extent dependent upon pathophysiological events occurring during reperfusion. The demonstration of unperfused regions after transient cerebral ischemia (Siesjö *et al.* 1968, Chiang *et al.* 1968, Cantu *et al.* 1969, Ginsberg and Myers 1972) focused attention on microvascular changes. Although it has been argued that the "no-reflow" phenomenon is not a common factor in the production of ischemic brain damage (Levy *et al.* 1971a and b) and, although it has been shown beyond doubt that irreversible neuronal damage may occur in the absence of circulatory impairment (Salford *et al.* 1973a and b, Siesjö *et al.* 1975a and b) postischemic microcirculatory disturbances must be considered as a possible mechanism, contributing to give irreversible cell damage in "transient" ischemia.

It has been shown by several investigators (Baldy-Moullinier 1970, Hosomatsu *et al.* 1973, Siesjö *et al.* 1975) that prolonged periods of reduction in blood flow may occur following severe cerebral ischemia. Although it is generally held that the cerebral metabolic rate for oxygen (CMR_{O_2}) is depressed during the initial postischemic recovery period (Lang *et al.* 1971, Siesjö *et al.* 1975, Hosomatsu *et al.* 1976) the reduction of cerebral blood flow may at least in some instances, represent a true hypoperfusion as indicated by a low cerebral venous oxygen content (Siesjö *et al.* 1975).

In previous communications we have shown that, in the rat, there is extensive recovery of cerebral cortex concentrations of high energy phosphates, glycolytic metabolites, citric cycle intermediates, associated amino acids, and ammonia after a 30 min period of complete cerebral ischemia irrespective of the type of anesthesia used (Nordström *et al.* 1977a). Following pronounced, incomplete ischemia of 30 min duration, however a considerable restitution of cerebral energy metabolism was obtained in deeply anesthetized (barbiturate 150 mg \cdot kg $^{-1}$ *i.p.*) but not in superficially anesthetized (70% N_2O) rats (Nordström *et al.* 1977b). Since both groups of animals showed a similar degree of metabolic perturbation during ischemia (Nordström and Siesjö 1977) it seemed likely that phenobarbital afforded protection by other mechanisms than by preventing energy depletion in cerebral cells. The objectives of the present investigation were (1) to study if the protective effects of barbiturate anesthesia could be attributed to a decrease in CMR_{O_2} during the initial recovery period, and (2) to investigate whether regional cerebral blood flow was distinctly higher in barbiturate-anesthetized animals during the recirculation period.

Methods

Adult male Wistar rats (270–300 g) with free access to tap water and rat pellets until operation were used in these experiments. The animals were anesthetized with either 70% N_2O or phenobarbital (150 mg \cdot kg $^{-1}$ *i.p.*) in the aortic cross-clamp group, anesthesia was induced with 2% halothane and, following tracheotomy, the animals were kept artificially ventilated on a gas mixture containing 70% N_2O and 30% O_2 . After tracheotomy the animals in the phenobarbital group were ventilated with 70% N_2 and 30% O_2 . All animals received tubocurarine chloride (1 mg \cdot kg $^{-1}$ *i.v.*) and heparin (100 IU \cdot kg $^{-1}$ *i.v.*). Rectal temperature was maintained close to 37°C by intermittent heating. A fall in brain temperature during the ischemic period was prevented by placing heating bulbs over the head at a pre-determined distance. Control values for regional cerebral blood flow (rCBF) are obtained in animals anesthetized with either barbiturate or phenobarbital. In these animals one femoral artery and one femoral vein were cannulated. In all animals made ischemic, both femoral arteries and both veins were cannulated. Arterial blood pressure was continuously recorded in all animals. Anaerobic sampling of arterial blood for measurement of P_{aO_2} , P_{aCO_2} , and pH was performed at regular intervals.

Postischemic Cerebral Blood Flow and Oxygen Utilization Rate in Rats Anesthetized with Nitrous Oxide or Phenobarbital

By

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Abstract

NORDSTRÖM C. H. and S. REINCRONA *Postischemic cerebral blood flow and oxygen utilization rate in rats anesthetized with nitrous oxide or phenobarbital* Acta physiol. scand. 101: 230-240.

The present experiments were undertaken to measure postischemic regional cerebral blood flow and oxygen utilization rate (CMRO₂) in rats anesthetized with either 70% N₂O or phenobarbital (10 mg/kg). In previous studies we have found that extensive restitution of cerebral energy metabolism after 30 min of complete cerebral ischemia irrespective of the type of anesthesia used. Following pronounced incomplete ischemia, however, comparable restitution of cerebral energy status was in deeply anesthetized (phenobarbital 150 mg/kg) but not in superficially anesthetized (N₂O) rats. The objectives of the present investigation were (1) to study whether postischemic cerebral blood flow was higher in barbiturate-anesthetized animals during the initial recirculation period, and (2) to test if the protective effects of phenobarbital previously observed could be attributed to a decrease in flow. In both groups of animals a considerable variability in postischemic rCBF was observed between animals. However, no signs of gross inhomogeneity in blood flow were found and no consistent differences in flow values between the two groups of animals were observed. Since the measured postischemic CMRO₂ were identical in both groups of animals and since cerebral venous oxygen contents were above normal, these results leave little support for the assumption that the present model of transient, incomplete ischemia (failure of recovery of cerebral metabolism (N₂O group)) is primarily due to impaired recirculation nor do they indicate that the protective effects of barbiturates is due to their ability to reduce rate of energy utilization.

During the last few years a substantial amount of evidence has accumulated, demonstrating that a majority of cortical neurons reconstitute energy metabolism and basal neurophysiological parameters after prolonged cerebral ischemia (15-60 min) (Häagen et al 1972, Häagen and Kleihues 1973, Ljunggren et al 1974 b, Folbergrova et al 1974, Hossmann and Hossmann 1974, Zimmermann and Hossmann 1975, Kleihues et al 1975, Marshall et al 1975, Nordström et al 1977 a). In contrast, clinical experience as well as a number of experimental studies have shown that following, for example cardiac arrest, signs of irreversible brain damage may develop even if the primary ischemia only last for 4-6 min (e.g. Cole and Cole 1956). Possibly the ultimate brain damage that is the end result is only a few mil-

rCBF ($\text{ml g}^{-1} \text{min}^{-1}$) in cerebral cortex (mean of six regions) and five subcortical regions in animals anesthetized with 70% N_2O or phenobarbital (150 mg kg^{-1}) during control conditions and after 5 and 15 min of ischemia. The values are means \pm S.E.

	Control N_2O (-5)	Ischemia N_2O		Control Phenobarbital (-3)	Ischemia Phenobarbital	
		5 min (-5)	15 min (-9)		5 min (-3)	15 min (-3)
I	0.34 ± 0.09	0.38 ± 0.17	0.34 ± 0.07	0.44 ± 0.04	0.37 ± 0.09	0.34 ± 0.03
II	0.62 ± 0.07	0.43 ± 0.11	0.30 ± 0.07	0.44 ± 0.04	0.38 ± 0.08	0.32 ± 0.04
III	0.76 ± 0.18	0.20 ± 0.07	0.22 ± 0.05	0.43 ± 0.06	0.24 ± 0.05	0.25 ± 0.02
IV	0.70 ± 0.09	0.22 ± 0.09	0.21 ± 0.05	0.39 ± 0.03	0.24 ± 0.06	0.26 ± 0.05
V	0.80 ± 0.13	0.09 ± 0.03	0.12 ± 0.04	0.44 ± 0.04	0.10 ± 0.02	0.12 ± 0.01
VI	0.83 ± 0.11	0.04 ± 0.01	0.04 ± 0.01	0.40 ± 0.04	0.04 ± 0.01	0.08 ± 0.03
VII	1.00 ± 0.08	0.05 ± 0.01	0.04 ± 0.01	0.42 ± 0.03	0.05 ± 0.01	0.04 ± 0.01

Results

Values for rCBF

Values for rCBF in animals anesthetized with either nitrous oxide or phenobarbital are in Table I. Since no differences in flow were observed between the 6 different regions the rCBF values are pooled. In animals anesthetized with nitrous oxide, flow was less in subcortical and brain stem regions. The values obtained are similar to previously measured with the same technique (Nilsson and Nordstrom 1977). In I, the results confirm those previously obtained with the autoradiographic techniques (e.g. 1955, Reivich *et al.* 1969). During phenobarbital anesthesia, cortical blood flow was reduced to 40-50% of normal. The values obtained are close to those measured by Kety and Schmidt technique (Nilsson and Siesjö 1975). The reduction of rCBF by phenobarbital anesthesia was most prominent in regions with a high proportion of matter (cf. Freygang and Sokoloff 1958).

During ischemia

I also gives the rCBF in animals subjected to 5 and 15 min of ischemia. There were two main findings. First, there was a very pronounced fall in cerebral cortex and caudate nucleus, somewhat less marked reduction in thalamus, mesencephalon and cerebellum, moderate reductions in pons and medulla oblongata. These results reflect the characteristics of the ischemic model, i.e. to drastically reduce flow to cortical regions while maintaining perfusion to brain stem regions. Second, there was a very small variation in rCBF between different cortical regions, and different animals. In the report by Eklöf and Siesjö

Incomplete ischemia

Pronounced, incomplete cerebral ischemia was induced with a technique similar to that described by Ekblöf and Siesjö (1972, see also Nordström and Siesjö 1977). Both common carotid arteries were cauterized, the vagi nerves were carefully separated from the arteries. Following operation the animals were kept disturbed for 20–30 min in physiological steady state. Incomplete cerebral ischemia was induced by rubber-coated clamps on both carotid arteries and reducing the arterial blood pressure to 30 mmHg. This was performed by using an automatically pressure-controlled syringe which extracted blood from the femoral artery and when necessary reinfused the previously extracted blood into one of the femoral veins. Recirculation of the brain was started by removal of both clamps and simultaneous infusion of blood. Within 1 min after the start of the recirculation, systemic arterial blood pressure was 100 mmHg in all animals and, within a few minutes, blood pressure reached 140–150 mmHg. During the initial recirculation period, amounts of sodium bicarbonate were given if needed and the ventilation volume was adjusted to correct for overrespiratory acidosis and hypercapnia, respectively. Arterial P_{O_2} and P_{CO_2} were kept above 100 mmHg and close to 40 mmHg, respectively.

Regional cerebral blood flow (CBF)

Regional cerebral blood flow was measured with ^{14}C -ethanol as a diffusible tracer (Ekblöf and Siesjö 1974). About 70% of the radioactive tracer was dissolved in physiological saline and injected intravenously for 30 s. Arterial blood was sampled in glass capillaries every 3 s during the infusion. Immediately after the last sample had been taken, 1 ml of a saturated KCl solution was rapidly injected into the carotid artery and the head was frozen in liquid nitrogen. The heads were kept at $-80^\circ C$ until dissection in a refrigerated glove box at $-14^\circ C$. Six cortical and 6 subcortical regions were dissected, weighed, added to scintillation vials and subsequently counted with the blood samples in a Nuclear Chicago scintillation counter. The partition coefficient for ^{14}C -ethanol was derived from Ekblöf *et al.* (1974).

rCBF was measured in animals anesthetized with either 70% N_2O or phenobarbital (150 mg/kg) during control conditions, after 5 and 15 min of ischemia and after 5, 15 and 30 min of recirculation. During ischemia of 15 or 30 min duration, in one group of animals (30 min of recirculation) arterial blood was taken from the superior sagittal sinus (see below) for measurement of the venous oxygen content and for calculation of arterio-venous differences in oxygen content.

Cerebral metabolic rate for oxygen

For quantitative estimation of postischemic cerebral blood flow and metabolic rate for oxygen, a modification of the Kety and Schmidt technique (1948) was used, as described by Norberg *et al.* (1974). Cortical venous blood was sampled in glass pipettes by puncturing the superior sagittal sinus previously exposed by a burr hole. The animals were saturated with ^{133}Xe via the insufflated mixture from a rubber balloon, containing about 10 μC of ^{133}Xe in either 70% N_2O and 30% O_2 or 100% O_2 . Saturation was started 15 min before induction of the ischemia and continued during ischemia and recirculation period. Desaturation was started by disconnecting the rubber balloon, artificial ventilation being continued with the original gas mixture. The rats were desaturated for 10 min. During this period arterial and venous blood samples were withdrawn at regular intervals for determination of the ^{133}Xe activity. Samples for determination of oxygen content in arterial and cerebral blood were taken at the end of the saturation period and after 5 and 10 min of desaturation. This method requires that (1) all parts of the tissue are saturated with ^{133}Xe before desaturation, (2) the insignificant contamination of venous blood from extracerebral sources, and (3) cerebral blood flow is constant during the period of desaturation. Therefore, 11 animals were discarded if the arterial and venous ^{133}Xe activities differed after saturation or desaturation, if the arterio-venous difference in oxygen content ($AVDO_2$) differed by more than 10%, or if the blood flow determination of ^{133}Xe activity and oxygen content was sampled in 50 μl glass capillaries with both ends drawn to thin tips. Blood pressure was kept constant during the measurements, using the automatically controlled syringe. Arterial P_{O_2} , P_{CO_2} were determined before and during desaturation.

The ^{133}Xe activity of the blood samples was measured in a Wallac gamma counter. CBF was calculated from the integrated arterial and cerebral venous desaturation curves (Norberg and Siesjö 1974). Tissue/blood coefficient for ^{133}Xe of 0.82. The arterial and venous oxygen contents were measured by the method of Fabel and Løbbens (1964) as described by Borgstrom *et al.* (1974). $CMRO_2$ was calculated as the product of CBF and $AVDO_2$.

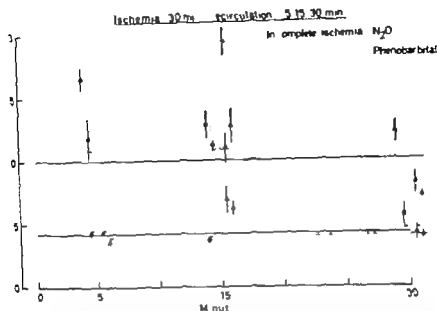


Fig. 1. Ischemic cortical blood flow (mean \pm S.E. for 6 cortical areas) obtained after 2, 15 and 30 min of recirculation following 30 min of preanesthetic isocapnic ischemia in animals anesthetized with 70% N₂O or phenobarbital (150 mg kg⁻¹). Present control values for cortical blood flow during isocapnia (1.00 \pm 0.08 ml g⁻¹ min⁻¹) and phenobarbital (0.42 \pm 0.03 ml g⁻¹ min⁻¹) anesthetics are

small differences were small in each animal. In a few animals a "reactive hyperemia" was observed during the first 5 min of recirculation. In the majority of animals, however, cortical blood flow was between 0.3 and 0.7 ml g⁻¹ min⁻¹ irrespective of the type of anesthesia.

Results demonstrate that, in animals anesthetized with phenobarbital, postischemic CBF values are close to those measured in the control situation. In contrast, rCBF in nitrous oxide-anesthetized animals was usually below control and, in some animals, the values were 30-40% of control.

Cerebral blood flow during recirculation after 30 min of ischemia

Figure 2 demonstrates cortical blood flow (mean \pm S.E. for 6 different cortical areas) after 2 and 30 min of recirculation in animals anesthetized with either nitrous oxide or phenobarbital. Again the variation in cortical blood flow between different animals was considerable. However, variations between different cortical areas were minor. Apart from the fact that there were some very high rCBF values also after 15 min of recirculation, the results are similar to those observed after 15 min of ischemia. Thus, in nitrous oxide-anesthetized animals there were some rCBF values that were only 30-40% of control.

Postischemic oxygen utilization

Normal rates of oxygen consumption in the brain, a reduction in CBF to below about 50% of control is usually considered inadequate for maintained energy production. It thus

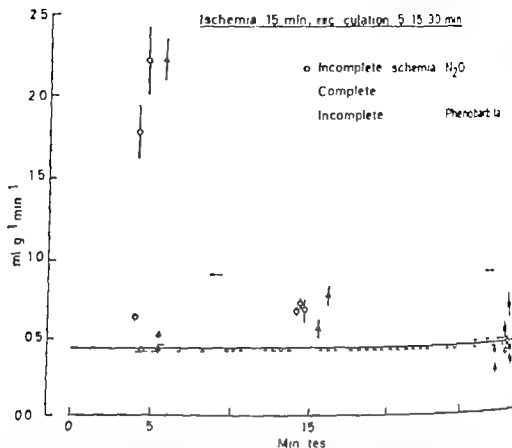


Fig. 1 Individual cortical blood flow values (mean \pm S.E. for 6 cortical areas) obtained after 5, 15 and 30 min of recirculation following 15 min of pronounced (incomplete) ischemia in animals anesthetized either 70% N_2O or phenobarbital (150 mg/kg). For comparison, cortical blood flow values obtained in 2 animals (70% N_2O) after 30 min of recirculation following 15 min of complete ischemia are also shown. Present control values for cortical blood flow during nitrous oxide (1.00 ± 0.08 ml g⁻¹ min⁻¹) and phenobarbital (0.42 ± 0.03 ml g⁻¹ min⁻¹) anesthesia are given.

(1972) the variations in cortical blood flow were pronounced. With the present slight modification of the experimental technique (a further reduction of blood pressure to 50 mmHg) such variations were not observed. However this does not exclude the possibility of inhomogeneity of blood flow at the capillary level. Third, in all animals, whether anesthetized with nitrous oxide or phenobarbital, cortical blood flow was reduced to about 0.05 ml g⁻¹ min⁻¹ to about 5% of control in nitrous oxide-anesthetized animals and about 10% in those given phenobarbital.

rCBF during recirculation. 15 min of ischemia

rCBF was measured after 5, 15 and 30 min of recirculation in animals anesthetized either nitrous oxide or phenobarbital. Fig. 1 gives the cortical blood flow (mean \pm S.E. for 6 different cortical areas) obtained in 27 animals. For comparison, the figure also shows postischemic cortical blood flow in two animals subjected to 15 min of complete cerebral ischemia (induced by elevation of the intracranial pressure above the arterial blood pressure). See Ljunggren *et al.* 1974 a, Nordström *et al.* 1977 a). As demonstrated in the figure, recirculation after 15 min of ischemia resulted in a rapid return of cortical blood flow to control values.

Table III gives the physiological parameters in these animals, as well as the arterio-venous differences for oxygen, the cerebral blood flow and the calculated CMR_{O_2} . The differences between CMR_{O_2} between animals anesthetized with nitrous oxide and phenobarbital are not significant. During physiological conditions, phenobarbital anesthesia reduces CMR_{O_2} by 40-50% when compared to nitrous oxide control values (Nilsson and 1975). Thus, in phenobarbital-anesthetized animals neither CBF nor CMR_{O_2} appears reduced during the initial recovery period following 15 min of ischemia. In contrast, animals under 70% N_2O had their CMR_{O_2} reduced to about 60% of control.

Discussion

In previous investigations we have described the postischemic cerebral blood flow (Hossmann *et al.* 1973, Hossmann and Kleihues 1973, Soyder *et al.* 1975, Nemoto *et al.* 1975), oxygen utilization rate (Lang *et al.* 1972, Soyder *et al.* 1975, Hossmann *et al.* 1976), and the correlation between postischemic hyperemia during the initial recirculation and functional recovery has been found (Hossmann and Kleihues 1973, Osborne and 1975, Soyder *et al.* 1975). However, since oxygen utilization rate may be considerably reduced during this period (Hossmann *et al.* 1976) it may be questioned whether this hyperemia is necessary for restitution.

A prolonged period of increase in cerebral blood flow frequently seen after ischemia has usually been explained by vasoparalysis due to lacticidosis (Zwetnow *et al.* 1968, Häggendahl *et al.* 1970, Kogure *et al.* 1970, Langfitt *et al.* 1965, Larsen 1966) but several factors are likely involved. Thus, it is known that tissue hyperosmolarity is an important mediator of edema in skeletal muscle (Mellander *et al.* 1967, Lundvall 1972) and in secreting endocrine glands (Lundvall and Holmberg 1974), and infusion of hypertonic solutions have been shown to affect vascular smooth muscles in brain (Johnston and Harper 1973, Wahl 1973). Certainly release of vasoactive substances during cerebral ischemia may also indirectly affect vascular tone. A reduction of postischemic cerebral blood flow has been attributed to a combination of pathophysiological changes: (1) aggregation of intravascular red corpuscles and increased viscosity (2) neuronal and glial swelling, and (3) capillary sheath swelling (Ames *et al.* 1968, Chiang *et al.* 1968, Cantu *et al.* 1969, Fischer and 1972, Fischer *et al.* 1975). Although it has been maintained that microvascular thromboses are not a common factor in the production of ischemic brain damage (Levy *et al.* 1975 a and b) they must nevertheless be considered since aggregation of blood corpuscles, leading to capillary obstruction, has been observed to occur *in vivo* (Eriksson and 1972) and since the no-reflow phenomenon has been described also in other animals (Flores *et al.* 1972, Franklin *et al.* 1974, Glaumann and Trump 1975). Altogether these complex mechanisms suggest the possibility of inhomogeneous blood flow following cerebral ischemia.

Most techniques used to measure cerebral blood flow are only valid if the flow is homogeneous. In the present investigation, this problem was approached by using two different methods for measuring cerebral blood flow and by combining the results with data obtained measuring cerebral energy metabolites (Nordström *et al.* 1977 a and b).

TABLE II Individual values for arterial and venous oxygen content, arteriovenous oxygen diff. cortical blood flow (mean \pm S.E. for 6 regions) after 30 min of recirculation, follow of ischemia in animals anesthetized with 70% N₂O or phenobarbital (150 mg kg⁻¹)

Anaesthesia	TaO ₂ μmol ml ⁻¹	TvO ₂	AVDO ₂	Cortical blood flow ml g ⁻¹ min ⁻¹
N ₂ O	10.45	9.26	1.19	0.56 ±0.09
	11.55	7.75	3.80	0.36 ±0.02
	8.97	7.80	1.17	1.21 ±0.09
	12.46	10.08	2.38	0.53 ±0.01
Phenobarbital	9.16	7.33	1.83	0.71 ±0.03
	10.41	9.94	0.47	0.81 ±0.09
	11.99	9.33	2.66	0.40 ±0.03
	10.78	8.18	2.60	0.42 ±0.06

became of crucial importance to find out whether or not postischemic blood flow was adequate for the energetic requirements. In order to study this problem, venous oxygen was measured and CMR_{O₂} was determined.

In animals exposed to 30 min of ischemia arterial and cerebral venous oxygen were measured following 30 min of recirculation. Individual values are given in Table II. There were some high arterial oxygen contents, probably due to hemoconcentration. However, since cerebral venous oxygen contents were higher than normal the result indicated that blood flow was insufficient for the requirements.

A quantitative measurement of cerebral blood flow and oxygen utilization rate was performed in 10 animals after about 30 min of recirculation following 15 min of

TABLE III Physiological parameters, cerebral blood flow (CBF) and calculated cerebral metabolic rate of oxygen (CMR_{O₂}) after 30 min of recirculation, following 15 min of ischemia in animals anesthetized with 70% N₂O or phenobarbital (150 mg kg⁻¹). The values are means

	N ₂ O (-5)	Phenobarbital (-5)
MABP mmHg	135 ± 4	130 ± 3
Temp, °C	37.1 ± 0.3	36.9 ± 0.1
Pao ₂ mmHg	117 ± 14	114 ± 14
Paco ₂ mmHg	38.0 ± 1.4	40.9 ± 4.4
pH	7.131 ± 0.063	7.226 ± 0.042
TaO ₂ μmol ml ⁻¹	11.2 ± 0.8	10.5 ± 0.3
TvO ₂ μmol ml ⁻¹	6.3 ± 1.2	6.1 ± 0.3
AVDO ₂ μmol × ml ⁻¹	4.8 ± 0.5	4.4 ± 0.3
CBF ml g ⁻¹ × min ⁻¹	0.60 ± 0.07	0.71 ± 0.07
CMR _{O₂} μmol × g ⁻¹ min ⁻¹	2.8 ± 0.1	3.1 ± 0.2

III gives the physiological parameters in these animals, as well as the arterio-venous for oxygen, the cerebral blood flow and the calculated CMR_{O_2} . The differences of CMR_{O_2} between animals anesthetized with nitrous oxide and phenobarbital is significant. During physiological conditions, phenobarbital anesthesia reduces CMR_{O_2} by 40–50% when compared to nitrous oxide control values (Nilsson and). Thus, in phenobarbital-anesthetized animals neither CBF nor CMR_{O_2} appears reduced during the initial recovery period following 15 min of ischemia. In contrast, after 70% N_2O had their CMR_{O_2} reduced to about 60% of control.

Discussion

Previous investigations have described the postischemic cerebral blood flow (Hossmann 1973, Hossmann and Kleihues 1973, Snyder *et al.* 1975, Nemoto *et al.* 1975) and oxygen utilization rate (Lang *et al.* 1972, Snyder *et al.* 1975, Hossmann *et al.* 1976), as well as a correlation between postischemic hyperemia during the initial recirculation and functional recovery has been found (Hossmann and Kleihues 1973, Osborne and Snyder *et al.* 1975). However, since oxygen utilization rate may be considerably reduced during this period (Hossmann *et al.* 1976) it may be questioned whether this is necessary for restitution.

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The techniques used to measure cerebral blood flow are only valid if the flow is homogeneous. In the present investigation, this problem was approached by using two different methods for measuring cerebral blood flow and by combining the results with data obtained during cerebral energy metabolites (Nordstrom *et al.* 1977 a and b).

Our previous results demonstrate that following severe, incomplete ischemia of 7 min duration, extensive recovery of cerebral metabolic state is observed in animals anesthetized with 150 mg/kg of phenobarbital, but not in those maintained on 70% N₂O. These results raise the following questions. First, is failure of recovery in nitrous oxide-anesthetized animals caused by vascular factors? Second, is the protective effect of phenobarbital due to reduction in metabolic rate to levels that can be covered by the (reduced) postischemic rate?

When recirculated following 15 min of ischemia most animals had regional cortical blood flow values between 0.3 and 0.7 ml/g/min. We recall that the lowest values represent only about 30% of control (see Results). These values are in reasonable agreement with those obtained with the Kety and Schmidt technique. Since only 3 out of 24 animals (70% N₂O) showed incomplete restitution of cerebral energy metabolism following a period of ischemia (see Nordström *et al.* 1977b) we may conclude that no cortex remained unperfused and that the low cerebral blood flow measured was sufficient to meet metabolic demands. This conclusion is supported by the high venous oxygen content measured and the reduced CMR_{O₂}, explains why the low CBF values encountered could still meet the energetic requirements. Since postischemic CMR_{O₂} did not differ between animals anesthetized with nitrous oxide and phenobarbital, the term "hyperemia" becomes ambiguous.

Following 30 min of ischemia, no animal anesthetized with nitrous oxide showed metabolic recovery while all animals given phenobarbital showed extensive restitution of energy state (Nordström *et al.* 1977b). The results obtained in the present investigation do not explain this difference. Thus, although several very low values for cortical rCBF were observed there were no consistent differences between the two groups of animals. Furthermore, all animals had cerebral venous oxygen contents above control after 30 min of occlusion. In other words, the present results leave little support to the assumption that the failure of recovery of cerebral metabolism in the present model of transient cerebral ischemia is due to impaired recirculation of ischemic tissue, nor do they indicate that the protective effect of barbiturates is due to their ability to reduce rate of cerebral energy utilization.

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Cardiac Receptors Activated during the Hypothalamic Defence Reaction

By

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Abstract

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Increases of arterial blood pressure, cardiac inotropy and venous return seen during the hypothalamic defence reaction are likely to lead to concomitant excitation of left ventricular receptors with nocomedullated inputs. The integrated efferent pattern of response resulting from the central interaction between the defence reaction and influences from the mentioned cardiac receptors was recently analyzed. These two, totally opposing influences on the circulation were then seen to interact in such a way as to produce optimized cardiovascular response with respect to increases in cardiac output and blood supply to the vital muscles. However, direct electrophysiological recordings from nocomedullated cardiac afferents during defence area stimulation have hitherto been lacking. The present experiments, performed on chloralose-anesthetized cats and utilizing electrophysiological recordings, clearly demonstrate that the left ventricular receptors are activated by the cardiovascular readjustments induced by the defence reaction. Defence area stimulation increased the activity of these receptors, which work within a very narrow low frequency range from 1.1 ± 0.3 impulses to 2.7 ± 0.7 impulses associated with rises in systolic blood pressure (afterload) and heart rate. Merely such receptor activation would induce considerable bradycardia and sympathetic inhibition, but particularly the latter bradycardia is centrally suppressed by concomitant defence area activation. The marked bradycardia often seen immediately upon interruption of the defence area stimulation is, however, probably to a great extent initiated from the excited ventricular receptors.

During the hypothalamic defence reaction, which is linked to situations of emotional excitement and even flight or attack, drastic readjustments of the circulation are seen. These cardiovascular changes, generally excitatory in nature, certainly lead to excitation of cardiovascular proprioceptors such as, e.g. the arterial baroreceptors (e.g. Hilton 1963). However, there is reason to assume that the cardiac left ventricular receptors with nocomedullated afferents also are activated during a full-fledged defence reaction (Wennergren, Lilander and Jørg 1976). This assumption seems reasonable in view of the cardiovascular changes during the defence reaction with a combination of arterial blood pressure rise, venoconstriction (Cobbold *et al.* 1964) and hence increased venous return as well as increased cardiac inotropy (Roven 1961), all factors known to activate these cardiac receptors (cf. Sleight and Widdi- 1965 Öberg and Thorén 1972, Muers and Sleight 1972, Thorén 1977).

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opened, reaching heart. The spikes were counted by spike counter device writing on the Grass tape. The technique has been described at length elsewhere. Öberg and Thorén (1972) and Thorén's papers should be consulted for details.

500 (Vitram), 1 000-1 700 IU/kg h.v. was used as anticoagulant. Once the stimulating electrode had been correctly positioned in the D.A. anastomosis flow was excluded and the heparin was neutralized with sodium chloride (Vitram), 10 mg per 1 000 IU heparin, in order to minimize bleeding in the area where afferents were detected for recording. During this recording the cats were conscious. 10 g gallamine triiodide (Flaxedil[®]), 3-5 mg/kg b.w., to decrease disturbing muscle movements.

Experimental procedures and treatment of data

1. D.A. was activated during continuous recording of cardiac receptor activity and with the hypothalamic shock response kept excluded from the earlier test stimulations. The hypothalamus was stimulated for 10-40 s and the stimulation was turned off once the defence reaction was fully developed. Expected increase of D.A. stimulation with concomitant recording of cardiac afferent activity was performed as test and the D.A. as a standardized power allowed to recover between each stimulation period. Electrical zones explored by the D.A. stimulation made simultaneous recording of cardiac afferent activity impossible. Instead, the receptor activity prevailing immediately after the D.A. stimulation was used, as taken to represent the reduced receptor activity. Several recordings were performed in each experiment, average values for each experiment are stated. These averaged data were used for calculating means \pm S.E. in the whole series. Each cat has been traced, with equal weight in the statistical analysis. Differences between pre- and poststimulation were tested by paired *t*-test. *P*-values ≤ 0.05 were taken to indicate significant difference.

Results

1 shows an example from a representative experiment. The D.A. is stimulated with Hz, 1 sec, 0.2 mA, stimulus parameters which earlier in the expt. were found to produce typical defence reaction with, in addition to the heart rate increase and blood pressure rise, also a skeletal muscle vasodilatation. A powerful poststimulation tachycardia was seen before administration of gallamine, which besides the curare-like effect is myotropic. The electrophysiological recording is from a non-medullated cardiac afferent in the receptor located in the left ventricle. It is seen that the cardiovascular readjustment during the defence reaction lead to a clearcut increase in the receptor activity which goes from about 0.4 imp/s to 2 imp/s. The activation of the receptor by the D.A. stimulation is clearly seen when comparing the 'silent' control neurogram (A) with that taken immediately after the end of the D.A. stimulation (B) where the receptor has started to fire regularly. Similar increases in activity at defence reaction were seen in all left ventricular receptors recorded from.

The results obtained from 5 receptors in 5 cats are summarized in Table 1. D.A. stimulation increased cardiac receptor activity from 1.1 ± 0.3 imp/s during the control situation to 0.7 imp/s. The difference is statistically significant. It should be pointed out in this context that these cardiac receptors with non-medullated afferents work within a very narrow low frequency range (Öberg and Thorén 1972). At electrical stimulation of afferent fibres, the steepest part of the frequency-reflex response curve occurs below 4 Hz (Öberg and Thorén 1972 a, Little, Wennergren and Öberg 1975). All the receptors on which Table 1 is based, were found in the left ventricle which is the main location for card

One question which then, of course, arises is to what extent these secondary influences may modify the primary centrally elicited defence reaction. When activated cardiac receptors with nonmedullated afferents normally give rise to a strong vagal bradycardia and a generalized inhibition of sympathetic tone to the vascular beds and the like. Thus, the reflex pattern normally produced by these cardiac receptors largely opposes the mainly excitatory pattern initiated from the hypothalamic defence area. An analysis of integrated response patterns resulting from interaction between the hypothalamic defence reaction and influences from cardiac receptors with nonmedullated afferents has not been performed (Wennergren, Lisander and Öberg 1976).

However, although the assumption that the left ventricular receptors are activated during the defence reaction may seem very reasonable, direct electrophysiological recordings from nonmedullated cardiac receptor afferents during defence area (D.A.) stimulation have earlier been lacking. The present experiments therefore have been performed to meet this need with electrophysiological techniques, whether and to what extent, left ventricular receptor activity is augmented during the defence reaction. The results confirm the hypothesis that the receptors are activated.

Methods

The experiments were performed on cats of either sex, anaesthetized with chloralose (30–50 mg/kg) after induction with ether. A tracheal cannula was inserted and the animals were placed on positive ventilation with 100 per cent O₂ with a tidal volume of 10–12 ml/kg b.w. The vago-sympathetic nerves and the carotid arteries were bilaterally cautiously freed in the neck. The arterial baroreceptors were left intact except the left carotid sinus which was functionally excluded due to cannulation of the common carotid artery (see below).

To counteract acidosis the cats were given a l. infusion (0.10–0.15 ml/min) of 10–20 mEq NaHCO₃ 100 ml dissolved in 10 per cent glucose solution (Haglund 1973). Acid-base balance was checked throughout and corrections were made by adjusting the rate of bicarbonate infusion.

A monopolar stainless steel electrode was placed in the hypothalamic D.A. using a Horsley-Clark apparatus. Stimuli were given by a constant current device as square wave pulses. The stimulus pulse was usually chosen to A 12.5 in the perforal region (cf. Jasper and Ajmonia 1961). A vasoconstriction resulting in a fall of flow resistance by 75 per cent or more with less than 10 latency was test stimulation at 0.2–0.3 mA, 1 ms duration and 80 Hz, was used as the criterion that the electrode was positioned in the D.A. The stimulation point was then determined histologically after formal perfusion and frozen sectioning.

When the correct position of the electrode had been ensured, the electrode was fixed to the skull with dental cement and the cat removed from the Horsley-Clark apparatus to facilitate the preparation for the electrophysiological recordings from cardiac afferents.

Recording

Recordings were made on a Grass Polygraph Model 7B–7D and on a BENI 3631 ultra sensitive (UV) recorder.

Arterial blood pressure, left intracardiac pressure and left atrial pressure were measured by Statham transducers (P23AC and P23DC) connected to catheters inserted through the left femoral artery, left carotid artery and right upper lung vein, respectively. Heart rate was recorded by a tachograph triggered by the rapid systolic blood pressure rise. Skeletal muscle blood flow was measured with a drop rate technique as the venous outflow from the calf muscles.

Carotid left ventricular receptor activity was recorded, with the thorax open, from non-medullated afferents dissected free in the right cervical vagus and displayed on the UV recorder. The receptors were localized to the left ventricle by their response to aortic occlusion and lack of response to normal occlusion (Swan-Ganz balloon catheter 93–110, 5F). The localization was confirmed by mechanical pressure

1. The responses to D.A. stimulation in terms of heart rate, systolic blood pressure (i.e. afterload), left atrial pressure and left ventricular receptor activity. Note the significantly decreased cardiac receptor activity in response to D.A. stimulation. Data based on average values from 5 cats. Mean \pm S.E. — none significant.

	Heart rate beats/min	Systolic arterial blood pressure mmHg	Left atrial pressure mmHg	Left ventricular receptor activity loop/s
at	212 \pm 3	151 \pm 11	5.6 \pm 0.4	1.1 \pm 0.3
>A. stim.	230 \pm 6	17 \pm 11	7.0 \pm 0.8	2.7 \pm 0.7
	5	5	5	5
pc	<0.05	0.001	n.s. (0.2)	<0.05

edicted by Wennergren, Lisander and Öberg (1976). III (in that study the interaction between the two influences was analysed and it was shown that a strong D.A. activation can completely suppress the otherwise powerful reflex vagal bradycardia elicited via the ventriculoceptors. In contrast, the inhibitory reflex influences from ventricular receptors on sympathetic vasoconstrictor and cardio-accelerator discharge seems to be unhampered to the extent that with the excitatory influences from the defence area, the net effect being largely a release of the reflex inhibitory and central excitatory influences on the peripheral sympathetic neurones.

In such a way the ventricular receptors can secondarily modify the primary centrogenic defence area influences, with the net result that two essentially oppositely directed influences on the cardiovascular system lead to an improved cardiovascular response in terms of a more efficient cardiac performance and enhanced blood supply to the skeletal muscles (Wennergren, Lisander and Öberg 1976).

When, however, a D.A. stimulation is turned off the tachycardia seen during stimulation is usually replaced by a powerful rebound bradycardia. This poststimulatory bradycardia is demonstrated in the sequence shown in Fig. 1 because of the gallamine administration causes vagal efferent blockade, but was seen before this administration. It has been pointed out that this bradycardia should be a baroreceptor reflex effect elicited by the increased arterial pressure and augmented heart rate. Due to the efficient suppression from the defence area of the baroreflex bradycardia, this reflex effect should, however, not develop when the D.A. stimulation is turned off (Hilton 1963). However, bradyarrhythmias following activation of hypothalamic pressor areas are still present after complete denervation of all arterial baroreceptors (Lisander, Jaju and Wang 1975), suggesting that the poststimulation dysrhythmias may also be reflexly induced via cardiac receptors.

The present demonstration that the unmyelinated cardiac ventricular receptors are indeed activated as a result of D.A. stimulation makes it likely that the poststimulation bradycardia is initiated from such receptors, the more so as they can elicit a much more potent vagal bradycardia than the baroreceptors (Öberg and Thoren 1973 b). The reflex bradycardia component from the ventricular receptors is suppressed during D.A. stimulation to as effectively as that from the arterial baroreceptors, and should therefore not appear when the D.A. stimulation is interrupted, as was also clearly shown by Wennergren, Lisander and Öberg (1976).

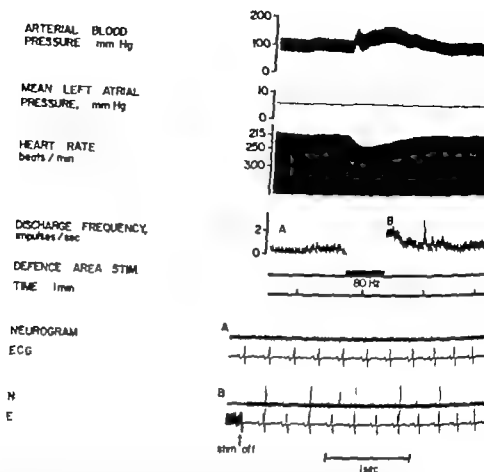


Fig. 1. The effects of D.A. stimulation (80 Hz, 1 ms, 0.1 mA) on the activity of a left ventricular signalling in a non-medullated afferent. Note that the defence reaction, with the typical increase in heart rate and arterial blood pressure, leads to a clear excitation of the ventricular receptor. The spike frequency increases from an average 0.4 imp/s before D.A. stimulation to about 2 imp/s immediately after the stimulation is turned off. Due to the prominent stimulation artefact the input of the nerve sample fiber in the control situation (A), where no spikes appear during the recording segment and immediately post D.A. stimulation (B), where the receptor has started to fire regularly at about 2 imp/s.

receptors with non-medullated afferents in the cat (Thorén 1977). The ventricular receptor activation was accompanied by a rise in systolic blood pressure (*i.e.* afterload), from 11 mmHg to 217 ± 11 mmHg and an increased heart rate from 212 ± 5 to 230 ± 6 beats/min. With regard to left atrial pressure which rather well reflects also left ventricular end-diastolic pressure, there was a tendency towards an increased preload, the pressure increasing from 5.6 ± 0.4 to 7.0 ± 0.8 mmHg, though this difference is not significant.

Discussion

The present experiments have demonstrated, with electrophysiological recordings, that left ventricular cardiac receptors, signalling in non-medullated afferents, are activated in the course of the cardiovascular readjustments induced by the hypotensive defence reaction.

Effects of Low Tissue Temperature on Peripheral Vascular Control Mechanisms

By

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Abstract

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Effects of low temperatures on vascular effects produced by adrenaline, noradrenaline and vasoconstrictor stimulation was studied in the feet of ducks. Observations are made on isolated preparations as well as on intact ducks with one or both feet immersed in ice-water. Isolated preparations are perfused physiologically and information on changes in vascular resistance obtained by direct measurement of flow changes during perfusion at constant pressure or by measuring changes in perfusion pressure at perfusion at constant flow. In intact ducks changes in blood flow are recorded as changes in digital skin tissue temperature. The study revealed that in the duck foot a relatively large fraction of the tissue blood flow is found in the arteries of the proximal part of the foot. During cooling of the foot the influence of vasoconstrictor nerve stimulation and low doses of adrenaline and noradrenaline on vascular resistance is gradually lost, and it is negligible at temperatures below 10°C. At these low temperatures the blood vessels stay dilated and blood flow through the web seems to be determined by the digital arterial blood pressure.

Local vascular changes induced by cold exposure of local tissues, particularly extremities, have been studied by many investigators (Lewis 1930, Grant and Bland 1931, Kræmer and Folkow 1948, Folkow *et al.* 1963). Initially the exposed vessels show constriction, followed by vascular relaxation if the cooling is continued. The mechanisms involved in the vascular response to cold are probably complex (Folkow *et al.* 1963), and their nature has not been fully explained.

Many birds are exposed to low ambient temperatures during winter. In such birds total body heat economy may require that blood flow through peripheral tissues like the feet be diminished at a time when high blood flow is necessary to prevent local tissue freezing. This raises the question of the relative importance of central and local vascular control in cold-exposed tissues.

The present paper reports on experiments into the efficiency of local vascular control mech-

During physical exercise similar cardiovascular changes are seen as in the defence reaction with increases in both inotropism and in pre- and afterload. It has been postulated that ventricular receptors are activated by the cardiovascular adjustments during exercise (Fr- and Widdicombe 1965, Thorén 1972) and the present findings are in line with such a postulate.

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lect of injection of noradrenaline (NA, 0.1 μ g, 0.02 μ g and 0.01 μ g) and of electrical stimulation (S) and nerve trunks (N) on perfusion pressure in the isolated duck foot perfused at 24°C. Note that stimulation of both artery and nerve trunks (S+N) in this preparation produced an effect that of the lowest dose of noradrenaline (0.01 μ g). The effect of stimulating the nerve trunks (N) only (S) alone is less.

caused a rise in perfusion pressure. Such stimulation also caused movement of the foot. The latter effect was prevented by adding α -tubocurarine to the perfusion fluid (5 ml), which in turn did not abolish the rise in perfusion pressure. A similar rise in pressure was also produced by periarterial stimulation. Simultaneous stimulation of the artery and nerve trunks gave a summation of the effects (Fig. 1). Injections of single doses of noradrenaline (0.01–0.1 μ g) into the perfusion fluid produced a dose-dependent rise in pressure. In one preparation, stimulation of the artery at 19°C increased the pressure to the same extent as did 0.01 μ g noradrenaline (Fig. 2, lower tracing). When the foot was cooled to 11°C doubling of the noradrenaline dose produced a response about the same as that caused by periarterial stimulation (Fig. 2, middle tracing). At still lower temperatures (4°C) the effect of periarterial stimulation was absent or negligible, and low doses of noradrenaline (0.01–0.02 μ g) had also no effect. Higher doses of noradrenaline (0.1 μ g) produced a weak but long-lasting rise in perfusion pressure (Fig. 2, upper tracing). During the preparation, it was shown that changes in the response to noradrenaline and electrical stimulation with cooling were reversible. The influence of temperature on the effect of nerve trunk stimulation was the same as that on the periarterial nerve stimulation. Independent of the effects of electrical stimulation and noradrenaline there was a rise in perfusion pressure with decreasing temperature. Cooling from 25°C to 5°C caused a rise of 10 mmHg. The rise was reversed by re-warming the preparation.

Temperature measurements in intact ducks. With one foot immersed in ice-water (0°C) up to the proximal end of the tarsal-metatarsal section and the other foot kept in air (22–23°C) the feet showed slow irregular temperature fluctuations which followed each other in time and direction, but were often different in magnitude. In the ice-water immersed foot the temperature measured subcutaneously on the medial side in the proximal part of the third tarsal phalanx usually fluctuated within the range of 2 to 9°C. This temperature range was reached within 5 min after immersion. The temperature of the foot in air usually stayed above 25°C, which was as in some birds only 2–3°C lower than the cloacal temperature of 40–41.5°C. Spontaneous bilateral temperature fluctuations were also seen when both feet were exposed to air at ambient temperatures (air or ice-water), but in that case the fluctuations of the two feet occurred within the same temperature range.

Perfusion pressure recording. Mean aortic blood pressure ranged between 135 and 220 mmHg. Perfusion pressure in the digital arteries generally followed the aortic blood pressure, but

animals in the feet of ducks at temperatures normally encountered during cold exposure. Vascular responses to sympathetic nerve stimulation and exogenous noradrenaline at various temperatures were studied in isolated perfused feet. Observations were also made on blood flow changes in the feet of intact ducks after intravascular administration of adrenaline or noradrenaline.

Materials and Methods

15 domestic ducks (*Anas boschas* b.w. 2.5–3.0 kg) were studied. All experiments were conducted during the winter months. The ducks were obtained from a local farmer shortly before being used. They had been in sheltered housing where air temperatures varied from about -5 to 10°C .

For perfusion of the isolated leg of the duck the foot artery was cannulated in the tarsal-metatarsal region after amputation of the leg. Amputation was performed under sodium pentobarbital anaesthesia (30 mg/kg b.w.). The polyethylene catheter used for the cannulation was filled with heparinized blood. The vascular bed was immediately flushed of blood. The two large mixed nerve trunks running along the main artery in the tarsal-metatarsal region were isolated to allow electrical stimulation. A bipolar stimulating electrode was secured around the artery distal to the cannulation site. The foot was perfused at constant flow by means of a peristaltic pump, using a physiological solution (Hank's solution) oxygenized with a gas mixture containing 95% O_2 and 5% CO_2 . The perfusion pressure was initially set to about 35 mmHg by adjusting the pump. Flow of perfusate at this pressure was 10–1 ml/min (corresponding to 32–40 ml per 100 g tissue). A higher initial perfusion pressure (60 mmHg) was tried, but this did not alter the nature of the results and had the adverse effect of increasing fluid accumulation in the foot.

During perfusion the foot was immersed in a water bath. The bath was gradually cooled from 37°C about 2°C by addition of crushed ice. The temperature of the perfusion fluid was adjusted to equal that of the bath.

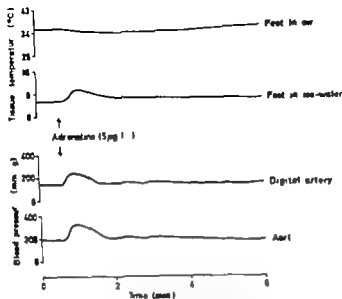
Electrical stimulation (10 V, 10 Hz, 5 ms duration) of nerve trunks and periarterial nerves, basally, was performed at various temperatures. The vascular response to these electrical stimulations was found to be of supramaximal intensity at all temperatures where a response could be elicited—as compared with the response to intravascularly administered noradrenaline. Noradrenaline, (0.01–0.1 μg) in a volume of 0.1–0.25 ml of physiological saline, was injected into the perfusion fluid (through the cannula) shortly before it entered the foot artery. Changes in perfusion pressure monitored by electromanometers reflected the magnitude of the vascular responses.

Experiments on intact ducks were performed under sodium pentobarbital anaesthesia (30 mg/kg b.w.) given i.v. supplemented by smaller doses as required. The ducks were suspended in a mesh net in a prone position, with their feet protruding to allow immersion in a mixture of crushed ice and water. Temperature of the room was $21 \pm 2^{\circ}\text{C}$. Aortic and digital arterial blood pressures were measured with electromanometers connected to polyethylene catheters. The aortic blood pressure was obtained by passing the catheter through the brachial artery. Digital arterial blood pressure was recorded from the digital artery in the proximal third of one of the digits. Drugs, dissolved in physiological saline, were administered through a catheter in a brachial vein in volumes of 1 ml over a period of about 5 s. Blood pressure was calculated as two-thirds of the diastolic pressure plus one-third of the systolic pressure. Cloacal and subcutaneous foot temperatures were determined by means of thermocouples. All data were recorded continuously over periods up to 6 h.

The vascular beds of the feet of two intact, anaesthetized ducks were studied in partially isolated form. A tourniquet was placed around the leg in the distal part of the tibia to stop blood flow to the foot and the main vein draining the foot was cut open on the medial side of the tarsus-metatarsus. The main foot artery was cannulated and perfused at a constant pressure of 40 mmHg with a solution similar to that used for perfusion of completely isolated feet, while the perfusate escaped through the opened vein. Changes in perfusate flow during cooling and rewarming of the foot was measured with an electromagnetic flow transducer placed on the inflow side of the perfusion system. The experiment was performed in the temperature range between 25°C and 5°C .

Results

Perfusion of isolated foot During perfusion at constant flow with both the foot and the perfusion fluid at 18 – 25°C , electrical stimulation of the nerve trunks running alongside



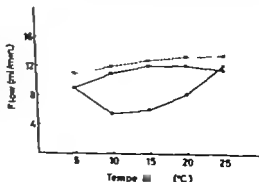
Effect of intravenous injection of adrenaline (5 µg) on mean aortic blood pressure, mean blood pressure in the digital artery (left foot, in ice-water), subcutaneous digital temperature of left foot, and a digital temperature of right foot (in air 22-23°C).

Discussion

provides evidence that in the ice-water immersed feet of ducks the blood flow in the web is mainly governed by the digital arterial blood pressure. It is moreover that rather steep pressure gradients and accordingly high vascular resistance exist in the arterial outflow vessels to the extremity.

Restrictor response of the vascular smooth muscle in the foot to both exogenous time and nerve stimulation was shown to subside progressively as the foot tissues cooled on ice-water immersion. Responses to noradrenaline can be elicited even at fairly high dose levels are required. Normal levels of adrenaline and noradrenaline

Changes in perfused flow during heating (●) and reversing (○) of perfused tissue with intact nerve supply. Dotted line shows temperature dependent flow in similarly perfused completely cut (Muller and Rie 1973). The separation of the flow change patterns reversing as reversal of that observed on cooling.



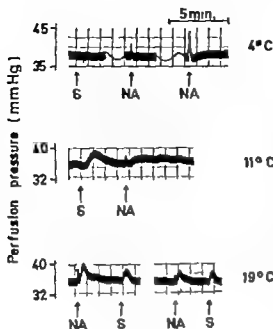


Fig. 2. Influence of temperature on the response to electrical stimulation of artery (S) to injection of noradrenaline (NA) in the perfused foot of the duck. At 4°C there was detectable effect of stimulation of the artery. A low dose of noradrenaline (0.02 μ g) also produced no effect, whereas a higher dose (0.1 μ g) produced a weak, but longlasting rise in perfusion pressure. At 11°C periaxillary nerve stimulation (S) had a small effect, and the effect of a low dose of noradrenaline (0.02 μ g) was fairly strong and longlasting. At 19°C noradrenaline (NA) produced marked effects at levels of both 0.01 and 0.02 μ g. The effect of periaxillary nerve stimulation (S) at the same rate was as that of the lowest dose of noradrenaline.

stayed 40–50 mmHg lower. Also with one foot immersed in ice-water and the other in air, the blood pressures in the digital arteries of the two feet remained within the same range.

Effect of drugs on blood pressure and foot temperatures. Adrenaline and noradrenaline doses of 2–10 μ g of the base given i.v. produced a marked rise in blood pressure both in the aorta and in the digital arteries. The pressure rise was accompanied by a rapid rise of 1–3 to 15°C in the subcutaneous digital temperature in the ice-water immersed foot while the opposite response of a decrease of 1 to 3°C or no detectable change in digital temperature characterized the warm foot (Fig. 3). As the blood pressure fell to the level prevailing before injection of adrenaline and noradrenaline the temperatures of the two feet gradually returned to their previous ranges. The effects of adrenaline and noradrenaline on blood pressure and foot temperatures remained after both cervical vagal trunks were severed, and they also persisted after administration of atropine (3 mg i.v.) and the β -adrenergic blocking agent propranolol (3 mg i.v.). After administration of the α -adrenergic blocking agents phentolamine (6 mg i.v.) or phenoxybenzamine (6 mg i.v.) both the blood pressure effects of adrenaline and noradrenaline and their effects on foot temperature were reduced. The α -adrenergic agents *per se* caused a foot temperature rise of up to 4–5°C when the initial temperatures were below 4°C and 30°C in the ice-water immersed foot and the foot in air respectively. At higher foot temperature levels the rise was less marked or absent.

Perfusion of foot with intact nerve supply. Cooling of duck feet perfused at constant pressure with the nerve supply intact showed a rapid decrease in flow from 25°C down to about 15°C. Between 15°C and 10°C the flow decreased more slowly, and from 10°C down to 5°C the flow increased. Rewarming from 5°C caused a further increase in flow until the temperature reached 15°C. From 15°C the flow decreased and at 25°C it stabilized slightly below the initial level. Changes in perfusate flow during cooling and rewarming as recorded in one of the experiments are shown in Fig. 4.

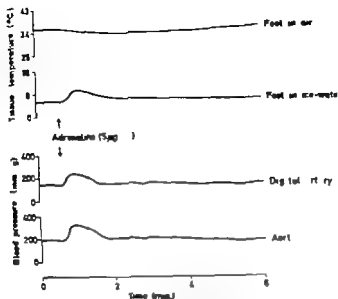


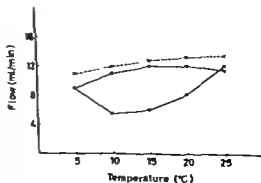
Fig. 3. Effect of intravenous injection of adrenaline (5 µg) on mean aortic blood pressure, mean blood flow in the digital artery (left foot, in ice-water), subcutaneous digital temperature of left foot, and systemic digital temperature of right foot (in air 22-23°C).

Discussion

This study provides evidence that in the ice-water immersed feet of ducks the blood flow through the web is mainly governed by the digital arterial blood pressure. It is moreover evident that rather steep pressure gradients and accordingly high vascular resistance exist along the arterial outflow vessels to the extremity.

The constrictor response of the vascular smooth muscle in the foot to both exogenous adrenaline and nerve stimulation was shown to subside progressively as the foot tissues were cooled on ice-water immersion. Responses to noradrenaline can be elicited even at 5°C, but fairly high dose levels are required. Normal levels of adrenaline and noradrenaline

Fig. 4. Changes in perfusate flow during cooling (●) and rewarming (○) of perfused left foot with intact nerve supply. Dotted line (---) shows temperature dependent flow changes in a similarly perfused completely denervated foot (Mallard and Rasts 1975). In the latter preparation the flow change pattern during rewarming was reversal of that observed during cooling.



In circulating blood are probably too low to exert a constrictor effect on the blood vessel the foot at tissue temperatures lower than 8–10°C.

In the completely isolated duck foot perfused at constant flow cooling from 25 to 5°C caused an increase in perfusion pressure of about 10 per cent, indicating a resistance increase of similar order of magnitude. Since the viscosity of water is about 70 per cent higher at 5°C than at 25°C (Weast 1973) the hindrance must be reduced by about 35 per cent. The reduction in hindrance at 5°C, as calculated from Poiseuille's equation, can be ascribed to cold-induced vasodilatation.

Flow changes seen during cooling and rewarming of the partly isolated perfused duck preparation were different from the flow changes previously observed in similarly perfused completely isolated feet (Millard and Reite 1975). The difference can be explained by the disappearance and reappearance of sympathetic vasoconstrictor tone in feet with intact nerve supply. Sympathetic nervous control seemed to be absent or very weak at temperatures below 8°C.

According to the present results any stimulus increasing the levels of adrenaline or noradrenaline in the blood or causing a general increase in sympathetic vasoconstrictor nerve activity will produce vasoconstriction mainly if not only in the warmer tissues. The resulting rise in blood pressure will cause a blood flow increase in the distal part of the water-immersed foot. This mechanism for local blood flow increase is not operable in tissues where vasoconstriction will affect the resistance along the entire foot vasculature. The main influence of sympathetic nerves on the blood flow through ice-water-immersed feet must be exerted on the larger arteries in the tarsal-metatarsal region where the temperature is higher than in the web. Maintenance of a blood supply adequate to prevent injury to the feet of ducks is therefore seemingly not due to humoral or neurohumoral mechanisms operating in the coldest tissues. Cold *per se* produces dilation of the blood vessels in the foot of the duck and the vessels remain dilated because they are unresponsive to sympathetic nerve stimulation and physiological levels of adrenaline and noradrenaline in circulating blood. The usefulness of controlling blood flow through cold-exposed tissues without depending on local control mechanisms is obvious. A locally induced vasoconstriction at low temperatures would be so long-lasting that damage would almost certainly follow. The only alternative would be to have an additional local vasodilating mechanism. Our data give no information on the relative contribution of nutritional blood vessels and arterial-venous anastomoses in the described responses. Fluctuations in the digital temperature represent changes in total flow to the foot or redistribution of blood flow within the foot.

The interpretation of our results as given above is compatible with previous reports. Folkow, Fuxe and Sonnenschein (1966) showed that blood vessels supplying skeletal muscle in the duck have a more dense sympathetic innervation and show a greater drop in pressure along the larger arteries than what is commonly found in mammals. If there is cold block of vasoconstrictor nerves in the peripheral small arteries and arterioles in a mammal, the increase of the vascular resistance will disappear which in turn may give rise to a pronounced local increase in blood flow. Consequently the tissue temperature will again rise, the cold block will be abolished, and the resistance reappear. This pattern may partly explain the 'hormet' reaction first described by Lewis (1930). In the duck consider blood resistance to flow as

is in the large arteries after cold blockade of the constrictor nerves supplying the more distal smaller ones. The typical hunting reaction which did not appear during our study, should, accordingly be absent or markedly reduced. Fluctuations in subcutaneous fat tissue temperature as demonstrated in the present work were small and irregular, both the cold and the warm foot were affected. Evidence has been presented that the hunting reaction is also absent in the feet of some arctic mammals (Henshaw Underwood Casey 1972), and that it is reduced in the hands of man acclimatized to cold (Krog *et al* 1971).

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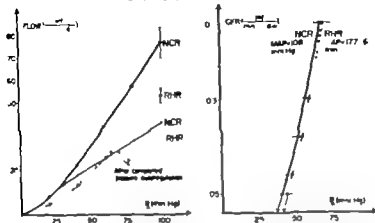
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3-4 MONTHS OF AGE



Left part shows the average relationship (± 3 S.E.) between arterial pressure (P_A) and flow (per g dry weight) in 9 pairs of maximally vasodilated NCR and RHR kidneys, both before and after the onset of "passive" autoregulation (see Methods). For each P_A level renal flow resistance per unit weight is clearly increased in the RHR kidney, both during 4-5 weeks had been exposed to an elevated MAP.

Right part shows the average relationship (± 3 S.E.) between P_A (after completed autoregulation) and filtration rate (GFR; ml/min/g dry tissue weight) for 9 pairs of NCR and RHR kidneys. Note the relationship, both reflects the structurally determined pre- to postglomerular resistance ratio, markedly shifted to the right in RHR, in striking contrast to the situation in SHR with established lesion (Folkow *et al.* 1977). — Correction for passive autoregulation (cf. left part) could make both much steeper but with little influence on their mutual relationships.

lesion as induced in 2½ month old NCR by clipping of the left renal artery leaving the right kidney (7½ kidney RHR). After 4-5 weeks, basal mean arterial pressure (MAP) was 177 ± 6 mmHg in the RHR, while paired experiments were performed on the isolated right RHR kidney, in comparison with fresh matched NCR, here MAP was 108 ± 3 mmHg.

RHR and NCR right kidneys were decapsulated and perfused with plasma substitute (Dextran 70 solution) during maximal dilatation and at room temperature to maximize tubular reabsorption (Folkow *et al.* 1977). Urine formation was continuously measured by sensitive weighing devices and was determined spectrophotometrically from the concentrations of Cr-EDTA in perfusate and urine. P -GFR relationships between P and GFR were determined by recordings of urine flow at various P_A starting here urine flow was just initiated. Then the GFR values were calculated, revealing in this reabsorption around 60 per cent, equal in both kidneys. The P -GFR relationships are then plotted the P levels after established passive autoregulation, representing the sum of the "true" pressure and the (mean pressure rise due to increasing afferent arteriole dilatation). Here filtration is enhanced at higher renal capillary pressures. Subsequently the pressure-perfusion flow relationships were determined, before (P taken exactly 5 min after the prompt flow shift) and after the full establishment of passive autoregulation. — Finally the dry weights of the kidneys are determined so that possible differences in size should not affect calculations of GFR and flow per unit weight.

1. Left part, shows the relationships between pressure (P) and perfusate flow/min/g kidney weight in RHR and NCR, both before and after established passive autoregulation during maximal vasodilatation. Obviously total renal vascular resistance per unit tissue weight was considerably higher in the pressure-exposed right RHR kidney compared with NCR one. However the intact RHR kidney had also increased its mass by hypertrophy

Structural Renal Vascular Changes in Renal Hypertensive Rats (RHR)

By

B. FOLKOW G. GÖTTBERG S. LUNDIN and S. E. RICKSTEN

Recently (Folkow *et al* 1977) total renal vascular resistance as well as the ratio between the pre- and postglomerular resistances were compared in paired perfusions of aortic vasodilated kidneys from spontaneously hypertensive rats (SHR) and matched normotensive controls (NCR). The comparison was performed both in 5 week old animals when SHR is in the phase of "borderline hypertension" and at 3-4 months of age when the SHR hypertension is fully established. Differences in the structurally determined pre- to postglomerular resistance ratio were estimated by comparing the respective relationships between aortic perfusion pressure (P_A) and glomerular filtration rate (GFR). If this ratio is increased in one of the animals, the corresponding P_A -GFR curve becomes parallelly displaced to the right along the P_A abscissa. If the glomerular filtration capacity is reduced as well, the steepness of the curve is correspondingly depressed. This technique has much in common with comparisons of the pre- to postcapillary resistance ratio and the capillary filtration capacity in other systemic circuits (cf Folkow *et al* 1974).

In the very young animals the SHR-NCR curves relating P_A to GFR were almost identical with the SHR curve just barely displaced towards the right. In contrast, in the 3-4 month old animals the SHR curve was displaced as much as 30-40 mmHg towards higher levels compared with the NCR one, but the curve steepness was still largely equal.

These results strongly suggest that the "triggering" neurohormonal pressor influences in SHR rapidly induce a structurally based vascular narrowing which in the kidneys is confined largely to the preglomerular vessels, responsible also of the wellknown renal blood flow autoregulation. In other systemic circuits this process of structural autoregulation involves the entire precapillary resistance compartment (Folkow *et al* 1974, 1977). The postglomerular resistance appeared to be, if anything, slightly reduced in SHR compared with NCR.

This rapid structural increase of the pre- to postglomerular resistance ratio in SHR implies a most efficient resetting towards hypertensive levels of the renal "long-term baroreceptor function" (cf Guyton *et al* 1974), but so far (up to 4 months of age) leaving the glomerular filtration capacity largely unchanged. The process is of the same principal nature as the resetting of systemic precapillary resistance, the "short term" barostats and the left ventricular function (cf Folkow, Hallböök and Norell 1977). The question arises whether exactly similar structural changes of the renal vascular bed occur in all types of hypertension. A comparison was therefore performed on kidneys from rats with renal hypertension (RHR) and NCR.

Effect of Dehydration on Renal Blood Flow in Dog

By

A. KIRKEBO and L. TYSEBOTH

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Abstract

and L. TYSEBOTH. *Effect of dehydration on renal blood flow in dog* Acta physiol. 1977 101 257-263

Dehydration on intrarenal blood flow was investigated in 11 dogs, using polarographic H_2 -gas densitometry for measuring local blood flow in outer cortex (ICF) and outer medulla (OCF). Dehydration was induced by 48 h water deprivation + 2-300 mg ethacrynic acid (EA) per dog the experiment. Compared to control group ($n=9$) ICF was markedly reduced to 40% (control 3.21 ± 0.64) whereas OCF 3.29 ± 0.80 ml/min/g was nearly unchanged (± 0.83). The ratio OCF/ICF was increased to 1.37 (1.11). Further dehydration by hypertonic dialysis for 3 h increased ICF to 60 ± 4 and further reduced OCF and ICF — almost significant — OCF/ICF-ratio. At Rct above 35 sudden and intermittent changes in local cortical blood flow occurred randomly at individual electrode sites, showing ischemic periods lasting for 1 to 5 min. Flow changes were observed in 13 of 14 cortex and were not accompanied by changes in RBF. In fact moderate dehydration causes greater reduction of ICF than of OCF. Severe dehydration in addition rose to patchy intermittent ischemia in both cortical layers.

in types of dehydration, ranging from severe salt to pure water loss, will in time decreased plasma volume, leading to reduced cardiac output, arterial pressure, renal flow and glomerular filtration rate. Dehydration and hypovolemia predispose oliguric renal failure and one might suspect that changes of intrarenal distribution of flow might be involved in addition to the reduction of local blood flow. — While renal flow distribution has been extensively studied in other low flow states such as acute hypotension and infusion of various vasoconstrictors, we are not aware of any data in dehydration, except for the preliminary report by us (Kirkebo and Tyseboth 1976) showing a greater reduction in inner than in outer cortical blood flow with periods of cortical ischemia.

Dehydration is most easily induced by a diureticum, and we have used ethacrynic acid which gives a shortlasting, but very high plasmaisotonic diuresis.

The purpose of our study was to investigate RBF and the local cortical blood flow distribution with the hydrogen washout method during different stages of dehydration induced by a combination of water deprivation, EA and hypertonic peritoneal dialysis.

(about 45 per cent) and the characteristic structural cardiovascular changes described by Lundgren (1974) were also observed in the present RHR.

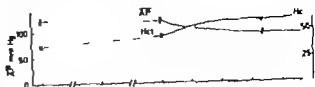
The right part of Fig. 1 shows the P_A -GFR relationships which are almost identical in RHR and NCR despite the fact that the RHR hypertension had lasted long enough (weeks) to fully complete the hypertrophic structural adaptation of heart and vessels. The nearly congruent RHR-NCR curves are in sharp contrast to the marked right-hand displacement of the P_A -GFR curve in SHR with established hypertension (Folkow *et al.* 1971).

The question arises why this is so. SHR hypertension seems to be initiated early by neurogenic mechanisms which predominantly affect the preglomerular vessels. In anything, lowering pressure at and beyond the glomerular level. However in RHR increased formation of angiotensin II seems mainly responsible for the pressure rise, and in the kidneys this substance preferentially constricts the postglomerular arteries (cf. Leyssac 1976). Consequently in the pressure-exposed RHR kidney transglomerular pressure is raised also in the postglomerular vessels. Since the local pressure is a main determinant of structural cardiovascular adaptation, both sets of renal vessels are likely to be structurally altered in RHR, and largely to a similar extent. While total renal resistance to flow per unit tissue weight is increased in the intact RHR kidney there is hardly any alteration of the pre- to postglomerular resistance ratio, in contrast to the increased ratio in established SHR hypertension, at least as long as no vascular wall hypertrophy is involved. This implies that the structural resetting of the barostat function may be quite differently organized in SHR and RHR, and in so doing helps to explain why hypertension is more readily reversed in RHR than in SHR (Lundgren 1974, Weiss 1974). From the largely unchanged pre- to postglomerular resistance ratio in RHR it follows that a MAP reduction does here *not* imply any undue lowering of glomerular filtration pressure, in contrast to established SHR hypertension where a full normalization of MAP may even eliminate GFR.

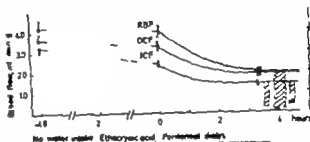
Supported by the Swedish Medical Research Council (14X-00016) and by a grant from the Faculty of Medicine, University of Göteborg. Thanks are due to Mrs Ulfa Axelsson and Mrs Birgitta Karlén for skilful technical assistance.

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and blood flow (\pm S.E.), for control dogs (9), dogs (14) exposed to pyrexia and ethacrynic acid 4 h before measurement together to hypertensive dialysis. Hatched bars only indicate periods with occurrence of periodic renal blood flow.



crises to 43.5 $\bar{A}P$ was 120 ± 10 mmHg (range 100–135 mmHg). RBF and OCF sized almost at the same level as in the control group presented above (Fig. 1). ICF : other hand was reduced to 2.40 ± 0.47 ml/min g, significantly lower ($p < 0.001$) OCF (3.29 ± 0.80 ml/min g) measured simultaneously. The ratio OCF/ICF had led to 1.37. The urine flow was low and urine osmolality about 1000 mosm/l at this : of dehydration, showing that the effect of EA had passed. The hydrogen washout were monoexponential, without any sudden changes in washout rate at individual odd sites.

very dehydration. The expts. were continued with repeated peritoneal dialysis using a toxic glucose Ringer solution. Urine flow ceased (below 0.01 ml/min) at Hct bout after 3 h of dialysis an extra fluid loss of approximately 0.5 l had been obtained and had increased to 60 ± 4 . $\bar{A}P$ fell to 95 ± 14 mmHg, while RBF decreased to 1.95 ± 0.70 ml/min g showing a rise in vascular resistance of about 60% (Fig. 1), including the rise blood viscosity.

th OCF and ICF were reduced to the same extent, i.e. about 60% of the local flow sized by moderate dehydration, thus maintaining the high OCF/ICF ratio of 1.35 in this severely dehydrated state.

t Hct higher than 55 shifts in local blood flow could be recorded as sudden changes in flow rate on the otherwise monoexponential hydrogen clearance curves. The flow could r from near control blood flow to zero flow at the same electrode site at consecutive clearance periods or at different time in the same washout period. Fig. shows an example re the H washout begins at a rate of 1.98 ml/min g, falls to 0.37 for about 1 min, to 1.20 ml/min g for another minute, falls to zero for 3 min and again increases to first washout rate of about ml/min g. In this single hydrogen washout curve 5 different flow rates were recorded, and the semilogarithmic plot shown in the middle panel is re at all different flow rates indicating abrupt changes in the local flow. As the zero line was very stable, flow could be determined down to very low level of H concentration, shown in Fig. 3 from 1 expt, the washout rate changed independently at different electrode

Methods

The investigation comprised one series of experiments on dehydration (induced by EA and/or dialysis). An additional series of 9 expts. performed simultaneously with the same technique was also. (Tyssbotn and Kirkebo 1975).

Before surgery the dogs were anesthetized by pentobarbital, 25 mg/kg b.w. and additional were given when needed. The dogs were intubated with a rubber endotracheal tube and breathed spontaneously through the whole experiment. Polyethylene catheters were placed in the saphenous vein, mean arterial pressure (AP) recording and in the saphenous vein for infusions. The kidney was retroperitoneally through a flank incision, and the renal artery gently dissected free for measure the total renal blood flow (RBF) by a Nycotron electromagnetic flowmeter. The flowmeter was clamped directly on a femoral artery. A polyvinyl catheter was inserted into the renal artery (Hend and Kirkebo) with the tip directed upstream, for infusion of the hydrogen saturated isotonic saline. The ureter was cannulated by a silicone rubber catheter for urine flow measurements.

3 L-shaped platinum electrodes were placed in outer half of the cortex with the sensitive tips 1-4 mm deep and 3 electrodes in the inner half of the cortex 5-8 mm deep. The distal electrodes were fixed to the renal capsule by two sutures and carefully covered by petrolatum. The area was closed by towel clips before measurements started. Hydrogen gas concentration around the electrode tip was determined polarographically at a polarization potential of +0.2 V vs. a 4% electrode placed subcutaneously on the hip (Aukland *et al.* 1973).

The amplified electrode current was recorded on a 6-channel recorder (Rikadeeli Kroyer Co. V B-64). For recording of a washout curve, 1-4 ml hydrogen saturated saline at 37°C was infused into the renal artery until constant H_2 -concentration was obtained at the different electrode sites. The course of $\frac{1}{2}$ -1 min. The infusion was then suddenly stopped and the washout curves were recorded thereafter plotted on a semilogarithmic paper. Blood flow in ml/min per g tissue was determined by the formula $0.693/T_{1/2}$ where $T_{1/2}$ is the half life in minutes of the hydrogen concentration in the urine. Urine osmolality was determined by freezing point osmometer (Advanced Instr.).

The kidney was excised at the end of the expt., drained and weighed, and the location of each electrode tip was carefully examined.

Dehydration by water deprivation EA and peritoneal dialysis The effect of dehydration on renal flow was determined in 14 expts. on 11 dogs (of both sexes) weighing 14-24 kg. The dogs were deprived for 48 h before the experimental measurements started. 4 h before the expt. 2-400 mg EA given per os. This produced a great plasma isotonic diuresis. The dogs had free access to dried milk until 18 h before surgery.

A silicone rubber catheter 6-7 mm diameter with many side holes was placed in between the ureters for infusion of hypertonic dialysing fluid consisting of 7% glucose in Ringer's solution and at 37-38°C. A small plastic cup with side holes was put between the intestines as a reservoir to facilitate removal of the dialysate.

At intervals of 30 min, 3-500 ml dialysate was infused and withdrawn after 20 min using a total of 1 500-2 000 ml dialysate. The hematocrit (Hct) was checked every 20 min and the rise in Hct was used to indicate the degree of hydration. Hydrogen washout curves were determined each 15 min during the period of increased dehydration. The peritoneal dialysis was continued until Hct had reached 45%. Thereafter hydrogen clearance was measured every 5-10 min for 1-2 h. T-tests were used for evaluation.

Results

Control measurements In a previously published study performed simultaneously and with the same technique in this laboratory (Tyssbotn and Kirkebo 1975), OCF and ICF in 10 dogs were 3.59 ± 0.85 ml/min/g and 3.23 ± 0.64 ml/min/g (mean \pm 1 S.D.) respectively, showing a ratio OCF/ICF of 1.11. RBF was 4.20 ml/min/g drained kidney weight and mean Hct was 37 and average AP was 122 mmHg in these dogs.

Dehydration by water deprivation and EA 48 h of water deprivation and 24 h after a total of 2-300 mg EA given per os, the body weight was reduced by about 10% and the average

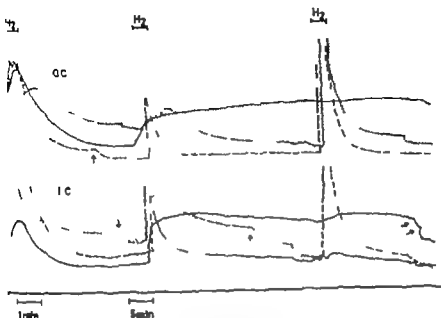


Fig. 1. Microcurves of 3 single electrodes in outer (OC) and 3 in inner (IC) cortex of a dog dehydrated for about 4 h, showing sudden changes in local blood flow. See text.

other. In some expts., however, the ischemic episodes were confined to only one side and were short-lasting, as further dehydration generally lead to lower flow and appearance of the intermittent changes.

Discussion

Effect of dehydration. To study the effect of dehydration it would have been desirable to measure local cortical blood flow repeatedly from chronically implanted electrodes. However, previous measurements have shown that the recorded local renal blood flow fell rapidly the first days after chronic implantation of platinum electrodes, while RBF was stable (Kirkebo and Tysebotn 1974). Therefore measurements with chronically implanted electrodes have not been performed on the same dogs before and after longlasting EA deprivation. As control for this series of expts. are used measurements done simultaneously with the same technique on 9 separate dogs (Tysebotn and Kirkebo 1975). The values from the chosen control material are slightly higher than control flow obtained in 5 dogs used for the study of the acute effect of EA injection, but the ratios OCF/ICF are identical. Both series agree well with control flow previously obtained by the H_2 short technique (Leyning 1971, Anklam *et al.* 1973, Kirkebo and Tysebotn 1974). In dogs dehydrated about 9% of b wt. by water deprivation and a dose of EA given the day before measurements, RBF and OCF were only insignificantly lower than in the control expts. even though H_{2O} had increased from 37 to 45. ICF fell to 74% of control, giving a rise in the ratio OCF/ICF from 1.11 to 1.37. The high urine osmolality showed that the effect

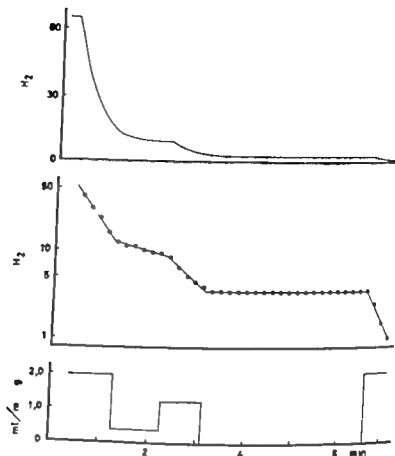


Fig. 2. H_2 washout curve of a single electrode (above) H replotted on semilogarithmic paper (panel) and the derived blood flow values (below), showing sudden, intermittent changes in flow. See text.

representing different zones. During the 3 washout periods presented in Fig. 3 at sudden increases in clearance rate (arrows) are observed on different electrodes in zones. Fig. 3 also shows zero or very low clearance in 5 more curves indicating zero at 2 electrode sites in the middle part and 3 in the right part of the panel. When in cover washout periods curves showed change from high to very low clearance rate a sudden gradual elevation of base line during the first washout period frequently could be used to detect the actual moment when the abrupt reduction in blood flow occurred.

Generally more than half of the electrodes showed marked shifts in H washout appearing at different electrode sites at different time, and the distribution of such changes occurred randomly in the outer and inner cortex. No synchronisations of the flow changes at different electrode sites was detected. Sudden changes in RBF was rarely recognised indicating that the observed changes in local cortical flow took place in relatively small regions probably not exceeding 10% of the total cortical volume. The duration of the ischemic periods varied greatly and unpredictably from less than 1 min up to 1 h. However, since the method does not permit continuous measurements, the beginning and end of the ischemia was often undetermined. Such patchy intermittent ischemia was observed at least at one electrode site in 13 out of 14 expts. and in some expts. involved all electrode sites at one

restriction is involved for instance at the level of the arcuate or interlobular arteries. In our experiments we have not been able to prevent the phenomenon by heparinization or by α -receptor blockade. However the variability and the fact that further dehydration usually lead to lower flow and disappearance of the intermittent changes have complicated systematic search for the mechanisms involved.

We are aware of only one previous report on flow changes comparable to those described in patients with hepatic cirrhosis and greatly reduced renal function Epstein et al. (1970) observed sudden changes in externally monitored ^{133}Xe washout curves. However from the published results it seems likely that the intermittent flow reduction in those cases involved the whole or greater parts of the cortex, whereas only small cortical regions seemed involved in the present experiments.

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of EA had passed and that the preferential reduction in ICF was due to an effect of dehydration. It should be noticed that Hct is lowered by bleeding and increased during dehydration and may be the key factor to explain why the ratio OCF/ICF is raised during dehydration and not during hemorrhage. Nybo Rasmussen (1976) has reported that in hydropic inner medulla was perfused with red cell poor blood. At the high Hct obtained by dehydration the red cell separation in preceding vessels might be less pronounced, so that Hct viscosity increased more in juxtamedullary than in outer cortical zones.

Severe dehydration induced by hypertonic peritoneal dialysis reduced both OCF and ICF to 60% of blood flow at moderate dehydration, and thus maintained a high ratio OCF/ICF. The flow reduction must be caused mainly by an increase in blood viscosity as Hct is raised to 60 thereby increasing total renal vascular resistance. Possibly the increase in viscosity (at least 1.5 times) (Chien *et al.* 1966) even covers that a slight vascular dilation take place. Since MacDonald (1976) reported that an increase of Hct caused no change in RBF this suggests that a vascular dilation has compensated the increased viscosity. Theoretically decreased AP and blood volume should induce a high concentration of vasoconstrictors at least of ADH, noradrenaline and angiotensin, therefore it is remarkable that a detectable vasoconstriction does not appear.

Intermittent and patchy flow reduction. In all previous work by the H₂ washout method we have observed essentially monoexponential washout curves and a relatively homogeneous blood flow distribution within the renal cortex. However, in states of severe dehydration Hct above 55 or during hind limb tourniquet shock, shifts in local flow occurred suddenly on single electrode sites without detectable changes in RBF. The records showed irregular patterns of intermittent and patchy reductions of blood flow ranging from control to zero flow and from less than 1 min up to 1 h. There is no indication of electrode movement as explanation for the abrupt changes in the washout rate, since a movement would induce an electrical spike, i.e. an abrupt elevation of the washout curve which within 1 min would back to a normal washout. Such spikes are never observed in connection to the abrupt shifts in washout rate. On the other hand, even if electrodes moved and detected a new washout rate it would just be another indication of areas in cortex with different washout rates.

Most of the washout rates are monoexponential down to less than 10% saturation, and the unilinear end of the desaturation curve depend on the change in zero line. In our studies the zero line occasionally are observed to increase at low flow rates, but this elevation is small compared to changes induced by the hydrogen concentration. Also, our studies hemorrhagic hypotension and vasoconstrictor infusions (Aukland *et al.* 1973, Kirkebo and Tyssebo 1974, Tyssebo and Kirkebo 1975) have never shown any abrupt changes in flow rate, even when RBF was very low (down to 15% of control flow) which furthermore suggest that the sudden changes in washout rate are not due to hyperreactivity only of the tissue immediately around the electrode tip.

Theoretically the electrodes use H₂ continuously but as shown by the minimal desaturation rate in Fig. 3 and by Tønder and Aukland (1974), the half life of H₂ current in a strictly limited cavity (dental pulps), was more than 1½ h after circulatory arrest.

The reversibility and the rapidity of the changes in local cortical blood flow

In the splanchnic vascular bed is the prime locus of action of endotoxins in dogs, the renal vascular bed constitutes the main target conduit in cats (Gilbert 1960 Kuida *et al.* 1961 Lüscher *et al.* 1964).

Material and Methods

Experiments were conducted on 9 cats of both sexes and weighing from 1.6 to 3.3 kg. The cats were anaesthetized with chloralose (100 mg/kg). They were intubated and ventilated with volume controlled respiration. The acid-base and blood gas state was determined by the Astrup technique, and corrections to physiological limits are made by adjusting the pulmonary ventilation or by giving concentrated sodium bicarbonate intravenously during the preparative phase. The oesophageal temperature was kept 38°C during the preparatory phase.

Electrodes were inserted into the thoracic aorta and the inferior caval vein via the femoral sheath. A left thoracotomy was performed, and sympathetic efferent postganglionic activity was recorded from the 3rd cardiac nerve. The fibre to be recorded was cut distally: the electrodes. The splanchnic activity is recorded from the cranial ends of these nerve fibres in the pedicle of the spleen and through left upper laparotomy. The aortic arch baroreceptor discharges were recorded on the left from the left splanchnic trunk.

Endotoxin *Escherichia coli* was given intravenously (1 mg/kg) within 10 min saline suspension (1 ml/kg). Recordings on the magnetic tape were started 1-2 min before the endotoxin injection and were carried continuously up to 5 min 20. Thereafter recordings, lasting 20 s, were made starting at 9 and 11 min. The number of nerve impulses per second as calculated with an automatic analyser (Nokia LP 4840, Ika Electronics, Finland). The means of the relative changes of discharge rates were calculated in all cats of the same fibre type for each 20-s period before and after the endotoxin injection. The means of these changes were calculated for the heart rate and the aortic and central venous pressures. The influence of changes was calculated with the Student's *t*-test.

For more detailed description of the methods see Häkkinen (1976).

Results

The average reactions of the parameters recorded are illustrated in Fig. 1 and Fig. 2 comprises a set of recordings from an individual experiment.

During the control period, the mean heart rate (\pm S.D. Fig. 1) was 181 ± 33 per minute. The systolic aortic pressure was 120 ± 38 mmHg and the diastolic one 94 ± 32 mmHg.

Following the endotoxin injection, the mean heart rate immediately decreased slightly (4%). It then increased to attain its maximum at about 3 min (220 ± 30 per minute). Subsequently following bradycardic transient, the mean heart rate decreased to control level.

The mean changes in the systolic and diastolic pressures were somewhat opposite to those of the heart rate. The lowest pressures, the systolic 75 mmHg and the diastolic 45 mmHg, coincided with the bradycardic transient (Fig. 1). Individually (Fig. 2), there was often, following the initial pressure drop one or two bouts of relative hypertension also discernible in the curves of mean changes (Fig. 1). At the end of the experimental period, hypotension still prevailed (Fig. 1 and 2).

The mean aortic baroreceptor afferentation (Fig. 1) decreased to 30 per cent of the control level. In some cats it ceased during the nadir of the aortic pressure (Fig. 2).

The cardiac sympathetic efferent discharges increased at 3 min after the injection but diminished during the rising aortic pressure after its first fall. During the ensuing secondary bout of hypotension, the cardiac sympathetic efferentation increased up to 140 times (Fig. 1).

Circulatory Reflex Responses during the Initial Stage of Feline Endotoxin Shock

By

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Abstract

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Cardiovascular and autonomic nervous responses to an injection of *E. coli* endotoxin were followed up to 15 min. in cats anaesthetized with chloralose and given artificial respiration. Within 60 s endotoxin induced drop of aortic pressure, with simultaneous cardiac acceleration and aortic venous hypertension. There was an associated almost complete cessation of the aortic arch bar afferentation. The cardiac sympathetic efferentation increased up to 1.4 times the control level maximum. The splenic sympathetic efferentation increased up to 10.6 times the control level at the 15 min period when the other parameters studied showed a trend to control level. The sympathetic autonomic system seems to be activated through cardiovascular receptors sensing hemodynamic changes touched off by endotoxin-induced release of vasoactive substances.

Key words. Endotoxin, autonomic nervous system, cardiac sympathetic efferent discharge, sympathetic efferent discharges, baroreceptor discharges

The release of vasoactive agents is likely to be the main cause of hypotension induced by endotoxin (Gilbert 1960 Hinshaw 1971). On the other hand, endotoxin has been proposed to be neurotoxic agents (Nagler and Levenson 1971) activating the sympathetic nervous system directly (Lillehei *et al* 1964). From the behaviour of the catecholamines and heart rate, in turn it has been concluded that endotoxins activate only the sympathetic (Egdahl 1959 a and b Nykiel and Glaviano 1961 Splink *et al* 1961 Levy and Blattberg 1967 Cavanagh *et al* 1970 Hall and Hodge 1971 Archer and Hinshaw 1975) but also the vagal system (Chien *et al* 1966, Levy and Blattberg 1967). The validity of this latter concept has been substantiated by our recent studies on the effect of endotoxin on cardiovascular reflex functions and circulation in dogs (Halinen, Hakumäki and Sarajas 1975 Halinen 1976). The purpose of the present report is to describe corresponding phenomena in cats. Such a comparative approach was considered useful since cardiovascular responses of cats to endotoxin seem to differ from those in dogs. For e-

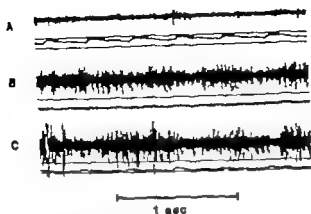


Fig. 1. Splenic sympathetic efferent discharge and aortic pressure curve before (A), 5 min (B) and 18 min (C) after bolus injection of endotoxin *E. coli* into cat. The horizontal lines on each tracing indicate aortic pressure levels at 70 and 9 mmHg.

The splenic sympathetic efferent activation exhibited a stepwise activation, the maximum, about 4-fold rise, became apparent at the end of the experimental period (Fig. 1). The relevant recordings obtained from an individual experiment are exemplified by Fig. 3.

The central venous pressure increased from the control level of 2.9 cm H₂O to 5.5 cm H₂O (0.05). This maximum level was attained 10 to 15 min following the endotoxin injection. Acid-base balance and blood gas state were determined before the endotoxin injection and at 11 and 21 min after it. At 11 min the mean arterial pH had decreased from 7.40 ± 0.04 (SD) to 7.33 ± 0.03 ($p < 0.01$), arterial P_{CO_2} from 30.4 ± 1.1 mmHg to 23.7 ± 1.1 mmHg (4.5) and the arterial P_{O_2} from 139 ± 15 mmHg to 102 ± 18 mmHg ($p < 0.01$) and the base bicarbonate from 21.3 ± 3.2 mmol/l to 16.2 ± 2 mmol/l ($p < 0.01$). At 21 min a recovery towards the control values was observed in all these parameters.

Discussion

Endotoxin evoked a drop of the arterial pressure within 60 seconds after the injection. This initial blood pressure fall is traceable to an endotoxin-induced pulmonary vasoconstriction being particularly pronounced in cats (Kulda *et al.* 1958; Kulda *et al.* 1961; Parrat 1973). The maximum pulmonary vasoconstriction ensues within a few minutes after endotoxin injection (Parrat and Sturgess 1975). Its relief may be responsible for the transient rise of the arterial pressure observed at 3-4 min following the endotoxin injection in the present study (Fig. 1).

The aortic arch baroreceptor discharges decreased, and in some cats completely ceased, with falling arterial pressure. After the endotoxin injection, the aortic pressure was below the threshold for the aortic baroreceptors (Sagawa, Kamada and Schramm 1974), but the discharge rate did not decrease lower than to about 30 per cent of the control level. No responses occurred in some cats. The changes of the

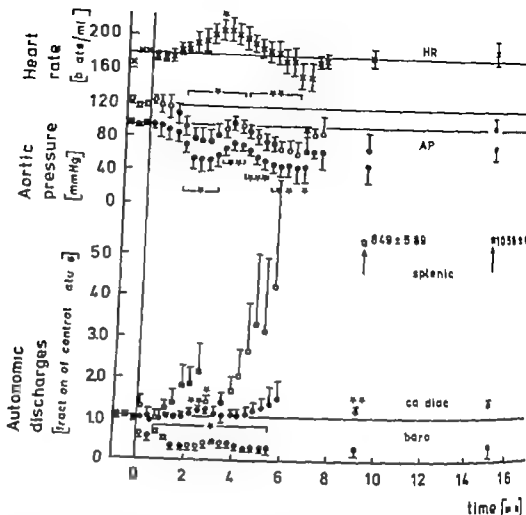


Fig. 1. Mean values (\pm S.E.) to the heart rate, aortic pressures (systolic and diastolic), the splenic (●) and cardiac (○) sympathetic efferent discharges, and the aortic arch baroreceptor (○) discharges in 9 cats given a bolus injection of *E. coli* endotoxin (1 mg/kg i.v.) at the zero time. Horizontal lines indicate control level of each parameter. Statistically significant changes are labelled with asterisks. (* $P < 0.01$, ** $P < 0.001$).

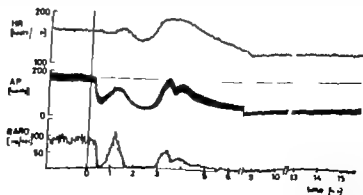


Fig. 2. Changes in the heart rate, aortic pressure and aortic arch baroreceptor discharge rates induced by a bolus injection of endotoxin *E. coli* L to a cat at zero time. Horizontal lines indicate the control level of each parameter.

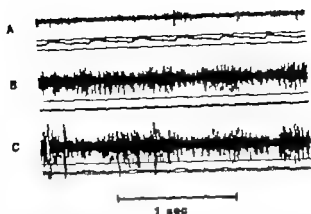


Fig. 1. Splenic sympathetic efferent discharges and aortic pressure curve before (A), 5 min (B) and 15 min (C) after bolus injection of endotoxin *E. coli* 055:58. The horizontal bars on each tracing indicate aortic pressure levels of 100 and 0 mmHg.

The splenic sympathetic efferent activation exhibited a stepwise activation: the maximum, about 4-fold rise, became apparent at the end of the experimental period (Fig. 1). The relevant recordings obtained from an individual experiment are exemplified by Fig. 3.

The central venous pressure increased from the control level of 2.9 cm H₂O to 5.6 cm H₂O ($P < 0.05$). This maximum level was attained 2 to 5 min following the endotoxin injection. Acid-base balance and blood gas state were determined before the endotoxin injection and at 11 and 21 min after it. At 11 min the mean arterial pH had decreased from 7.40 ± 0.04 (D.) to 7.33 ± 0.03 ($P < 0.01$), arterial P_{CO_2} from 30.4 ± 1.1 mmHg to 23.7 ± 1.1 mmHg (4.5%) and the arterial P_{O_2} from 139 ± 25 mmHg to 102 ± 18 mmHg ($P < 0.01$) and the plasma bicarbonate from 21.3 ± 3.2 mmol/l to 16.2 ± 2 mmol/l ($P < 0.01$). At 21 min a recovery towards the control values was observed in all these parameters.

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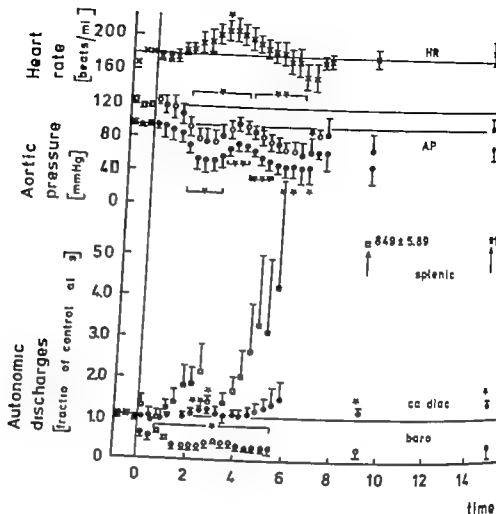


Fig. 1 Mean values (\pm S.E.) for the heart rate, aortic pressures (systolic and diastolic), the sympathetic (●) and cardiac (○) discharges, and the aortic arch baroreceptor (○) discharges given a bolus injection of *E. coli* endotoxin (1 mg/kg i.v.) at the zero time. Horizontal lines indicate the control level of each parameter. Statistically significant changes are labelled with asterisks (* $P < 0.01$, ** $P < 0.001$).

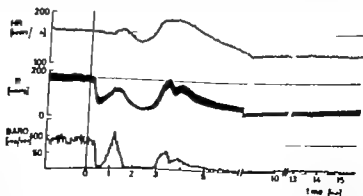


Fig. 2 Changes in the heart rate, aortic pressure and aortic arch baroreceptor discharge rates under a bolus injection of endotoxin *E. coli* L- to cat at zero time. Horizontal lines indicate the control of each parameter.

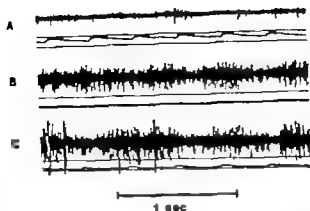


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The central venous pressure increased from the control level of 2.9 cm H₂O to 5.6 cm H₂O ($p < 0.05$). This maximum level was attained 2 to 5 min following the endotoxin injection. Acid-base balance and blood gas state were determined before the endotoxin injection and at 11 and 21 min after it. At 11 min the mean arterial pH had decreased from 7.40 ± 0.04 (SD) to 7.33 ± 0.03 ($p < 0.01$), arterial P_{CO_2} from 30.4 ± 1.1 mmHg to 23.7 ± 1.1 mmHg ($p < 0.01$) and the arterial P_{O_2} from 139 ± 25 mmHg to 102 ± 18 mmHg ($p < 0.01$) and the plasma bicarbonate from 21.3 ± 3.2 mmol/l to 16.2 ± 2 mmol/l ($p < 0.01$). At 21 min a recovery towards the control values was observed in all these parameters.

Discussion

Endotoxin evoked a drop of the arterial pressure within 60 seconds after the injection. This initial blood pressure fall is traceable to an endotoxin-induced pulmonary vasoconstriction being particularly pronounced in cats (Kulda *et al.* 1958, Kulda *et al.* 1961, Parrat 1973). The maximum pulmonary vasoconstriction ensues within a few minutes after endotoxin injection (Parrat and Sturgess 1975). Its relief may be responsible for the transient rise of the arterial pressure observed at 3–4 min following the endotoxin injection in the present study (Fig. 1).

The aortic arch baroreceptor discharges decreased, and in some cats completely ceased, with falling arterial pressure. After the endotoxin injection, the aortic pressure was below the threshold for the aortic baroreceptors (Sagawa, Kumada and Schramm 1974), but the mean discharge rate did not decrease lower than about 30 per cent of the control level. However only minor hypotensive responses occurred in some cats. The changes of the

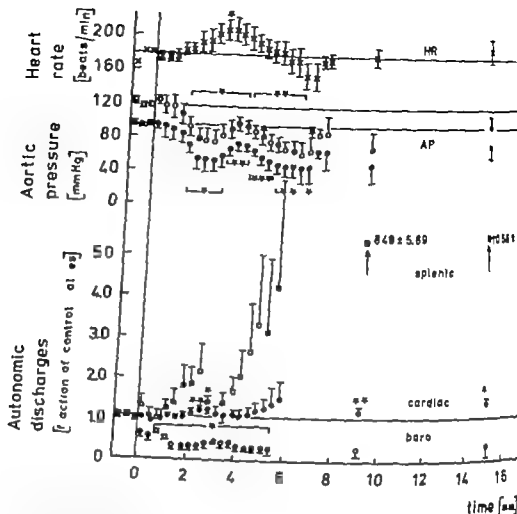


Fig. 1 Mean values (\pm S.E.) for the heart rate, aortic pressures (systolic and diastolic), the splenic and cardiac (●) sympathetic efferent discharges, and the aortic arch baroreceptor (○) discharges in a given a bolus injection of *E. coli* endotoxin (1 mg/kg i.v.) at the zero time. Horizontal lines indicate control level of each parameter. Statistically significant changes are labelled with asterisks (* $P < 0.01$, ** $P < 0.001$).

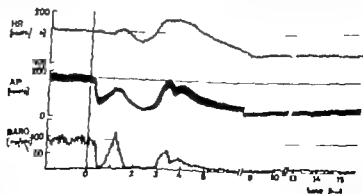


Fig. 2 Changes in the heart rate, aortic pressure and aortic arch baroreceptor discharge rates induced by a bolus injection of endotoxin (*E. coli*) to a cat at zero time. Horizontal lines indicate the control level of each parameter.

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sympathetic discharge rates inversely followed those of the arterial pressure. The cat sympathetic activation, however, seemed to be delayed when compared to the pressure fall. This agrees with the observation of Parrat (1973) who reported a decrease of cardiac contractility and its recovery within 1-3 min of the initial endotoxin-induced hypotension.

The heart rate began to increase after a slight initial reduction and reached its maximum at 3 min after the injection. An initial bradycardic reaction has been observed in cats in response to endotoxin injection (Parrat 1973), and this bradycardic episode is followed by tachycardia (Parrat and Sturgess 1975). The initial bradycardia may arise from the anaesthetic pentobarbital for in pentobarbitalized dogs bradycardic responses to endotoxin have been frequently reported (Levy and Blattberg 1967). A vagal activation in cats with endotoxaemia was observed in a previous study (Halinen, Hakumäki and Sarajä 1976; Halinen 1976). If the phenomenon exists in cats, it might explain the decreasing heart rate after 3 min despite of the sympathetic activation in the present work.

The splenic efferentation gradually increased up to about 10-fold. Like the carotid afferent discharge the splenic efferentation mirrored the arterial pressure changes. The splenic and cardiac sympathetic fibres are known to respond in a parallel way to the baroreceptor input. The gain of the baroreceptor-sympathetic system to the spleen was normally 2-fold greater than the gain to the heart at aortic pressure levels below 100 mmHg. This is more than that in cats under pentobarbital (1.6-fold) reported by Nishimaru and Irisawa (1971) within an aortic pressure range of 100 to 150 mmHg.

Slight but significant changes towards metabolic acidosis and a fall of arterial pressure occurred in the cats of the present study. These phenomena have been reported also earlier in dogs and cats (Hardaway *et al.* 1961; Dedichen 1972; Parrat and Sturgess 1975; Halinen 1976). They are ascribable to decreased cardiac output (Gilbert 1960) and to changes in the pulmonary circulation leading to pulmonary edema in response to endotoxin (Halinen *et al.* 1961).

The average cardiovascular responses to endotoxin in cats were delayed when compared to those in dogs under morphine-chloralose anaesthesia (Halinen 1976). Also the cardiac sympathetic efferentation was activated substantially later in cats as well. Despite of the distinct tachycardia elicited in dogs and the transient tachycardia in cats, the responses were nevertheless uniform in general. The splanchnic sympathetic activation presumably occurs also in dogs as shown by intestinal blood flow measurements by Liljeblat *et al.* (1964) and Dietzman *et al.* (1973).

The hemodynamic responses to endotoxin are likely to result from the release of vasoactive amines (*cf.* Greenway and Stark 1971; Lefer 1973; Elin and Wolff 1976) into the circulation in response to endotoxins. Possibly the hemodynamic changes secondarily activate the sympathetic nervous system through cardiovascular receptors. The sympathetic system is nevertheless in competition with potent vasodilator substances, and the increased sympathetic drive may actually lead to a further impairment of the peripheral circulation.

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Influence of Muscle Length on the Force-Velocity Relation of K^+ -contractures in Smooth Muscle from Rabbit Urinary Bladder

By

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Abstract

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Force-velocity relations of K^+ -contractures of longitudinal smooth muscle from rabbit urinary bladder were studied by isotonic quick release at 37°C. In order to minimize the influence of parallel elastic elements the study was limited to the rising part of the length-tension curve. The force-velocity data fitted well Hill's equation. The in situ length of the strip at a bladder volume of 10 ml is called L_{10} . This length was 50% of that at which maximum active tension is developed. At L_{10} V_{max} was 0.29 muscle lengths per sec. and it was estimated to be 0.36 length/s at optimum length. Constant b in Hill's equation had a value of 0.052 L_{10}/s and it was unaffected by length changes over the interval $0.69 L_{10} - 1.44 L_{10}$. At L_{10} P was 0.17 P_0 . In the interval given above, a/P decreased with increasing length in proportion to the increase in P_0 , indicating that a was also length independent. According to Hill's equation $[V - b(P - P_0)]/P$ should increase in proportion to $(P - P_0)$ when the muscle length is increased if a and b are constants. For a linear relation was found at shorter lengths but at lengths close to or at the length for maximum active tension, V increased more than $(P - P_0)$. Two possible explanations were considered, firstly that b increased, and secondly that the load on the contractile element could be less than P due to an influence of the considerable tension in the parallel elastic element at these lengths. The series elastic element of the active muscle amounted to 3-4% of the muscle length when released to zero tension.

The relationship between force and shortening velocity in muscle is an important characteristic of the contractile machinery. It can be described mathematically by Hill's equation (Hill 1938) $v(P+a) = b(P - P_0)$ which represents a rectangular hyperbola with asymptotes at $v = -b$ and $P = -a$, and a P axis intercept at P_0 , the isometric force. Hill's early work on frog skeletal muscle indicated that the constants a and b which can thus be obtained from the force-velocity relation by purely mechanical experiments, reflected directly the rate of energy liberated during shortening (mechanical work + shortening heat). Later investigations have cast doubt on this direct relation between thermal and mechanical constants (see e.g. Simmons and Jewell 1974). However the force-velocity relation is still of great importance in view of its mere mechanical implications. It illustrates directly the power output from the muscle as a function of load and determines e.g. the rate at which the active muscle may shorten in response to a change in load.

aching of skeletal muscle to sarcomere lengths above $2.0 \mu\text{m}$ causes the isometric \dot{x} to decline, whereas the maximal shortening velocity (V_{max}) remains almost un-
 ped (Gordon, Huxley and Julian 1966). On the rising phase of the length-tension curve,
 changes roughly in proportion to P_0 . The constants a and b in Hill's equation are in-
 upon unaffected by length changes (Abbot and Wilkie 1953). For heart muscle P
 \dot{x}_{max} have been found to increase proportionately with length on the ascending part
 length-tension curve while the constants a and b remain unchanged (Nilsson 1972).
 e mechanics of smooth muscle has been studied a great deal over the last few years.
 tively smooth muscle behaves as other muscle types in that the force-velocity relation
 is fitted to the Hill equation, but V_{max} is much lower than in striated muscle (see e.g.
 ens, Kroeger and Mehta 1969, Gordon and Siegman 1971 a, Herlitzky and Murphy
 Peterson 1974, Hellstrand and Johansson 1975). There is, however, not enough informa-
 concerning the question of how the force-velocity curve in smooth muscle is affected
 nith changes although some studies have been done (Gordon and Siegman 1971 a,
 et al. 1975). It seems that further systematic investigation is necessary.
 e aim of the present expts. on smooth muscle from rabbit urinary bladder was to study
 ffect of length on V_{max} , P and the constants a and b in Hill's equation. This preparation
 tains low resting tension for quite large changes in length (Uvelius 1976 b). The expts.
 performed using the isotonic quick release method on K^+ -activated strips. Some of the
 have been reported in preliminary form (Uvelius 1976 a).

Methods

Six of either sex weighing 2.5-3 kg were killed by cervical fracture and the urinary bladder was
 and out, cleaned and transferred to Ca^{2+} -free Krebs solution (composition in mM: NaCl 122,
 NaHCO_3 15.5, K_2HPO_4 1.19, MgCl_2 1.19 and glucose 11.5). After 1 h in this medium at 4°C ,
 seemed to cause complete relaxation, the bladder was filled to 10 ml with the Ca -free solution and
 most of the longitudinal smooth muscle coat was marked out, dissected free and transferred to the
 bath in the apparatus described below. As in the earlier studies (Uvelius 1976 a, b) the length of the
 bath bladder volume of 10 ml will be referred to as L_0 .
 e recording apparatus was essentially the same as described earlier (Johansson 1973) except that the
 is now as fixed with small metal clips at both its ends. One clip was connected to an isometric force
 duct and the other to a light lever (equivalent mass 150 mg with the muscle clip attached). The lever
 tical photoelectric displacement transducer and could be clamped or released by an electromagnetic
 and stop. By means of a spiral spring the load on the lever could be varied over the range of 0 to 10^{-6} N.
 nus of the expts. the initial oscillations of the lever after release were reduced by letting it move in
 the slot containing a drop of oil of variable viscosity. In other expts. the lever was undamped. There
 no differences in the force-velocity characteristics of the muscles as obtained from recordings with
 without damping. The output from the apparatus was recorded on a linear direct-writing oscillograph
 with M.V.4.
 e muscle was allowed to accommodate for 1 h in a physiological salt solution of the following composi-
 tion in M: NaCl 120, KCl 6.0, MgCl_2 1.2, CaCl_2 2.2, glucose 11.5 and tri(hydroxymethyl)aminomethane
 (see Birc, Sigmund Chemical Co) 35. The solution had been titrated with HCl at 37°C to a pH of 7.4
 as in the following be referred to as "Na-tris, 2.5 Ca^{2+} ". After the accommodation period the muscle
 transferred to a solution with the same composition except that CaCl_2 was omitted (Ca -free Na-tris).
 taneous mechanical activity then rapidly disappeared. Preload was kept at about 2-4% of maximum
 tension unless otherwise stated. After 5 min the muscle was transferred to K -tris, 2.5 Ca^{2+} /
 L. With the same composition as Na-tris, 2.5 Ca^{2+} except that all NaCl was replaced with equimolar
 amounts of KCl . In this solution the muscle develops steady contracture. The preparation was kept in

SHORTENING

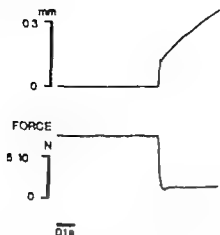


Fig. 1 A typical recording of an undamped release. The muscle is released from 7.5 to 1.4 mV (panel). After the release the muscle recoils rapidly after some inertial oscillations it begins to shorten at a slower rate (upper panel).

in 0.1 M K^+ tris, 2.5 mM Ca^{2+} for 5 min and was then transferred to Ca^{2+} -free, Na-tris for 5 min which produced complete relaxation. This time schedule was then maintained throughout the experiment. Quick releases against various loads were made during the steady contractions. All solutions were bubbled with O_2 and kept at 37°C .

Results are given below as mean values \pm S.E.

Results and Comments

Fig. 1 shows an original recording obtained for a quick release against a moderate load during a steady contraction in 0.1 M K^+ tris, 2.5 mM Ca^{2+} . Immediately after the release the muscle recoils rapidly and this event is followed by slower shortening.

The low preloads used in this study made it possible to leave the parallel elasticity for consideration. According to Johansson, Hellstrand and Uvelius (1977), the length of the series elastic component (SE) followed by a velocity transient lasting 50–75 ms and finally a slower phase in which the contractile element is fully adjusted to the load. In order to avoid the influence of the velocity transient, shortening velocity was measured 100 ms after the release. To measure shortening velocity at a fixed time like this may lead to the fact that the measurements are taken at different lengths of the contractile element. These differences did however not exceed 3% of the muscle length in this study. Between P and P₀ the difference in length did not exceed 1%. The recoil of the SE element was in all cases 3–4% of the muscle length when the fully activated muscle preparation was released to zero load.

The relation between load and velocity at all lengths studied was found to fit well with Hill's (1938) equation. By plotting the experimental data as $(1 - P/P_0)/V$ vs. P/P_0 and applying linear regression analysis, V_{\max} and the constants a and b could be calculated. The solid line in Fig. 2a shows the configuration of the hyperbola that fitted best to the data for one muscle at L_{10} when examined by such linearization of Hill's equation. After the series of releases at L_{10} the length of the muscle was passively changed and the whole sequence was repeated at $L_{0.77}$. The lower curve of Fig. 2a shows the force-velocity relation at $L = 0.77 L_{10}$. It is seen that V_{\max} expressed in mm/s is lower at the shorter muscle length. V_{\max} velocity is

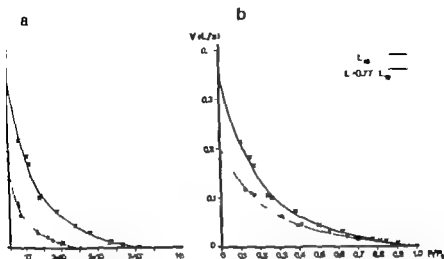


Fig. 2. F-V relations for one muscle studied at L_0 and $0.77 L_0$. The hyperbolas that fitted best with the data examined by linearization of Hill's equation are shown in the figure. 2 shows shortening rate expressed as mm/s and force as mmHg. In 2 b shortening rate is expressed as the actual muscle length times, force expressed relative to the isometric tension times the respective length.

fraction of the actual muscle length per second, a lower value of V_{max} was obtained at the shorter length (Fig. 2 b). In this figure P is normalized as P/P_0 . In most experiments it was possible to obtain 2 complete force-velocity curves from the muscle. For the 8 muscles studied in this study V_{max} at L_0 was $0.29 \pm 0.03 L_0/s$.

A earlier study has shown the effects of length on isometric contracture force in the rabbit muscle of the bladder (Uvelius 1976 b). To see if change of muscle length would affect V_{max} (expressed as mm/s) and P to the same extent, the quotient between the values at the greater and the shorter length examined in the individual experiment of the present study was divided by the corresponding quotient for V_{max} . If the relative change in P was paralleled by the same relative change in V_{max} the result should be 1. A figure of 1.04 ($n = 8$) was obtained suggesting that V_{max} is, if anything, more sensitive than P to variations in length over the range examined here (see further below).

The value of the constant b was found to be 0.032 ± 0.008 muscle lengths per second at L_0 (Fig. 3). The effect of length changes on constant b is shown in Fig. 3 A. The value of L_0 for the individual muscle was set to 100% and the values obtained at other lengths expressed as percent of the L_0 value. It is seen in the figure that there was no systematic change of b in response to variations in length. The mean length of the stretched muscles was $1.33 L_0$ ($n = 5$) and for these the constant b became $0.054 \pm 0.007 L_0/s$. It thus appears that b is essentially independent of the degree of stretch for the length interval studied in this preparation.

The constant b and P are both dependent on the cross-sectional area of the muscle and it is therefore appropriate to examine the quotient between the two values. In the present study b/P was found to be 0.17 ± 0.02 ($n = 8$) at $L = L_0$. When the muscles were stretched,

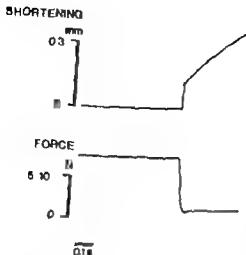


Fig. 1. A typical recording of an undamped load release. The muscle is released from 7.5 to 14 N (lower panel). After the release the muscle recoils rapidly after some inertial oscillations it begins to shorten at a slower rate (upper panel).

K tris, 2.5 Ca^{2+} for 5 min and was then transferred to Ca -free, Na tris for 5 min which produced complete relaxation. This time schedule was then maintained throughout the experiment. Coid trials against various loads were made during the steady contractions. All solutions were bubbled with O_2 and kept at 37 $^{\circ}\text{C}$.

Results are given below as mean values \pm S.E.

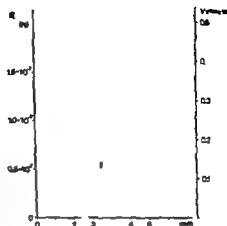
Results and Comments

Fig. 1 shows an original recording obtained for a quick release against a moderate load during a steady contraction in K tris, 2.5 Ca^{2+} . Immediately after the release the muscle recoils rapidly and this event is followed by slower shortening.

The low preloads used in this study made it possible to leave the parallel elasticity out of consideration. According to Johansson, Hellstrand and Uvelius (1977) the length of the series elastic component (SE) followed by a velocity transient lasting 50–75 ms and finally a slower phase in which the contractile element is fully adjusted to the load in order to avoid the influence of the velocity transient, shortening velocity was measured 100 ms after the release. To measure shortening velocity at a fixed time like this may be that the measurements are taken at different lengths of the contractile element. These variations did however not exceed 3% of the muscle length in this study. Between P and P₀ the difference in length did not exceed 1%. The recoil of the SE element was in all cases negligible at zero load.

The relation between load and velocity at all lengths studied was found to fit well with Hill's (1938) equation. By plotting the experimental data as $(1 - P/P_0)/V$ vs P/P_0 and applying linear regression analysis, V_{max} and the constants a and b could be calculated. The solid line in Fig. 2a shows the configuration of the hyperbola that fitted best to the data for one muscle at $L_{1.2}$ when examined by such linearization of Hill's equation. After the series of releases at L_1 the length of the muscle was passively changed and the whole sequence was repeated. The lower curve of Fig. 2a shows the force-velocity relation at $L \sim 0.77 L_{1.2}$. It is seen that V_{max} expressed in mm/s is lower at the shorter muscle length. Also, if velocity is expressed

Results from one experiment showing $P - P_0$ (circles) and V (crosses) as functions of muscle length. V is the shortening velocity against load P was held constant (in this exp. $0.3 \cdot 10^{-4}$ N). V increases in proportion up to muscle length 55 mm. Above this length V increases $\propto P - P_0$ as for this muscle 4.4 mm. (cf. details see text).



of 0.29 length/s at L_{10} . The maximal active tension at optimal length is 13 N/cm^2 (1976 a, b). These differences between smooth muscles from various sources may be due to differences in gross tissue structure (presence of other tissue components such as connective tissue, inhomogeneity of cell orientation, variations in size of extracellular space) but they may also reflect biochemical and ultrastructural variations in the myosin systems (see Rüegg 1971).

In the urinary bladder muscle both V_{max} and P increase with muscle length up to where P is maximal. Further stretching decreases P whereas V_{max} is unaltered (Gordon, Huxley and Julian 1971). Due to the considerable passive tension of the strips of urinary bladder at lengths less than $1.8 \cdot L_{10}$ it was only possible to study the force-velocity relations on the rising part of the length-tension diagram. For length changes between 69 and 144 per cent of L_{10} both V and P changed to about the same relative extent. This was seen both in recordings of the force-velocity curves at different lengths and by the fact that the shortening velocity at a given load, P , was proportional to $(P - P_0)$. At larger lengths, V was found to increase faster than $P - P_0$ (see Fig. 4). The reason for this greater increase is not clear. It may be related to changes in one or both of the parameters a and b but it is also possible that the force carried by the contractile element is simply less than the afterloaded P at these lengths due to an influence of the parallel elasticity. It is thus possible that the true relation between V_{max} and P is linear also at these lengths. At L_{10} V_{max} was 0.29 muscle length/s. At this length, P is 2.5 times P_0 at L_{10} (Uvelius 1976 a, b). V_{max} at the optimal length may then be estimated to be about 0.36 length/s. The above experiments suggested that a and b are constants independent of length in the range between 69 and 144 per cent of L_{10} . For b this is also supported by the results shown in Fig. 5A. The constant b was $0.052 \pm 0.008 \text{ L}_{10}/\text{s}$ at L_{10} and $0.054 \pm 0.007 \text{ L}_{10}/\text{s}$ at $1.33 \cdot L_{10}$. The reported values for b in smooth muscle may be exemplified by the following data: 0.035 for cat intestinal muscle at 30°C (Mehs 1971), 0.02 for hog carotid artery (Herbby and Murphy 1974) and 0.05 for guinea pig taenia coli at 36°C (Mashima and Honda 1969).

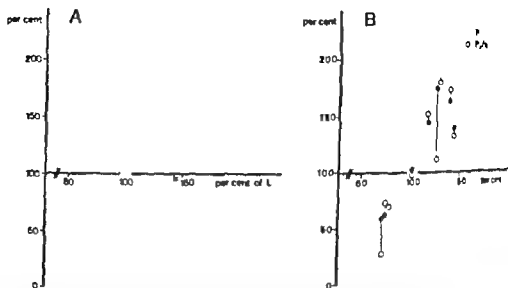


Fig. 3 A Effect of length changes on constant b in Hill's equation. B Influence of length changes on isometric force (P_0) and P_0/a . The values of the variables were set to 100% at L_{10} and the values at other lengths were expressed as percent of the L_{10} values. P and P_0/a seem to be affected by length changes in the interval studied ($0.69 L_{10} - 1.44 L_{10}$), whereas no systematic effect on b is seen.

a/P was found to decrease. In order to see if this decrease at longer lengths was due to the increase of P_0 , a plot of P_0/a and P was made. The value of both at L_{10} has been set to 100% in Fig. 3 B. If a itself is independent of length then P_0/a and P should be affected to the same degree by changes of the muscle lengths and according to the results this seems to be the case.

The Hill equation can be written as $V \sim [b/(P+a)] (P - P_0)$. Then, if a and b are independent of length as the above experiments indicate, the velocity of shortening against a load, P , will be proportional to $(P - P_0)$ when P is varied by changes in the muscle length. In a separate set of experiments the preparations were released against the same load during contractions elicited at several different lengths. Fig. 4 summarizes results from one of these experiments. It is seen that with increasing length V and $(P - P_0)$ increase proportionately up to about $1.25 L_{10}$. Above this length V increases faster than $(P - P_0)$. This means that up to about $1.25 L_{10}$ V is a linear function of $(P - P_0)$ and that above this value $b/(P+a)$ increases. This finding will be commented upon in the discussion.

Discussion

Maximal shortening velocity varies greatly between different smooth muscles. Strips of hog carotid artery show a V_{max} of only 0.12 muscle lengths per second at 37°C (Hellebrand and Murphy 1974) whereas values of 0.74 lengths/s have been obtained in the rat portal vein (Hellebrand and Johansson 1975). On the other hand the preparation of hog carotid artery develops high isometric tension ($P = 18$ N/cm²) whereas the rat portal vein reaches 3-4 N/cm² vessel wall or 8-10 N/cm² muscle layer (Johansson 1976). Strips of longitudinal smooth muscle from rabbit urinary bladder have, according to the present study,

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The absolute values of the constant a of a preparation is dependent on the tension development which is in itself dependent on cross sectional area. It is common therefore to use a/P instead of a . At the optimal length, a/P is 0.24 for cat intestinal muscle at 30°C (1974) 0.18 for hog carotid artery (Herlihy and Murphy 1974) and 0.21 for dog taenia (Stephens *et al.* 1969) both at 37°C. In our preparation P_0/a and P varied to the same with the preparation length (Fig. 3 B) and thus a was also a constant within the interval studied. This is in accordance with the findings of Abbot and Wille (1958) on skeletal muscle Nilsson (1972) on papillary muscle and Gordon and Siegmán (1971) on rabbit taenia coli. The quotient a/P is, in contrast to a not constant it decreases at greater lengths as P increases. Thus no characteristic a/P can be given but at the value is 0.17 ± 0.02 . The optimal length for active tension development is, as determined earlier (Uvelius 1976 a, b), about $2.0 L_0$ at which the muscle develops about 2% more active tension than at L_0 . Hence a/P at this length should be about 0.07 if the results indicate a length independent constant. Gordon and Siegmán (1971 a) found no significant change in a/P with length in their taenia coli preparation but their values at length were smaller than in this study.

In summary it can be said that the value of b found in this study is in good agreement with those obtained for other smooth muscles, whereas the estimated a/P at optimum is considerably smaller. It could be possible that a at the optimal length for this preparation might be greater than at the lengths studied or that the high resting tension present in some of the studies by others could influence a . Finally the mode of activation could influence the results. Hellstrand and Johansson (1975) and Murphy (1976) have noted that activation by high solution as in this study increases the curvature of the force-velocity relationship in comparison to that found in tetanic activation and this would imply a lower a in contractures.

The maximum extension of the series elastic component was 3-4% of the muscle length a value which is lower than the ones reported earlier for vertebrate smooth muscle. For other preparations vary between 20% for rabbit taenia coli (Gordon and Siegmán 1971) and 4.3% for rabbit mesotubarium (Meiss 1975). These considerable variations may in part be due to different amounts of injured tissues in the preparations. It is also possible that differences in apparatus compliance might contribute. That there exist true differences in SE behaviour for different smooth muscles is, however, shown by Johansson (1977). In their study a comparison was made between the SE stiffness for rabbit bladder smooth muscle and rat portal vein. The SE element of the bladder preparation were found to be about 3 times stiffer than that of the portal vein at any force level. The preparation used in the present study is histologically very homogeneous. The nuclei of the cells have the same direction as the whole muscle and this might be one reason for the stiff SE but further study on this point is necessary.

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and Albrecht 1975). Another indirect method, Xe-clearance, requires a homogeneous flow to be valid (Kety 1951). So far there is no conclusive evidence for a homogeneous flow throughout the testis. The use of radioactive microspheres would offer an advantage over the technique mentioned above, since the method can be applied in a surgically intact rat and it requires no homogeneously perfused vascular bed. In addition, this method has been shown to be suitable to measure flow in hyperperfused tissues (Janson and Albrecht 1975). The technique was modified for use in small laboratory animals by Rudolph and Hey (1967) and was recently applied to the rat (Bruce 1976). In the literature there is no full report on its use for testicular blood flow measurements.

The purpose of the present study was to set up a technique for measuring testicular blood flow in the rat by use of radioactive microspheres and to compare this method with the newly used Xenon-133 clearance technique and with direct measurement of spermatic vein outflow. While there is no information available on the distribution of blood flow in various segments of the testis, an additional purpose of the study was to perform such measurements.

Materials and Methods

Sixty 80 male rats of the Sprague-Dawley strain (Møllegaard-Hansen, Ejby Denmark), weighing between 300 and 400 g, were kept in controlled environment. Food and water were available *ad libitum*. Animals were anesthetized by sodium pentobarbitone (Nembutal, Abbott, U.K.) in a dose of 40 mg/kg p. given as single injection. During each exp. the rat was kept supine on heating

Preparation for testicular blood flow measurement

For measurement 15 animals were laparotomized and the intestines as gently pulled upward and to the left and covered with a cloth soaked in warm saline. By this procedure the right spermatic artery and vein were exposed. By use of a polyethylene catheter (PE 30, Intramedic, Clay Adams, U.S.A.) the spermatic vein was cannulated at least 5 mm from its entry into the caval vein. No ligation of the spermatic vein was made to the cannulation as necessary since the PE 30 catheter fits very tightly into the vein and no leakage of blood was possible. Care was taken to avoid manipulation of the spermatic artery. Blood flow measurement was started 2-3 min after the cannulation. The catheter 3-4 cm in length, was kept in a fixed position with its open end at the level of the caval vein. The spermatic venous blood was collected in pre-weighed glass tubes for one-minute period after which the testicular artery was ligated through a small made subcutaneous incision (see Fig. 1). Immediately following the ligation, the spermatic venous flow was measured for another one-minute period. The blood volume was calculated by use of the weight of rat body blood, 1.05. By subtracting the blood volume collected after ligation of the testicular artery from the total spermatic venous outflow testicular blood flow was calculated. During whole experimental period the blood losses were substituted for by giving an equal volume of Macroderm (AB, Sweden) into the external jugular vein.

Xenon-133 clearance. Xenon-133 dissolved in sterile saline was obtained from AB Atomenergi, Stockholm, Sweden. Approximately 20-40 μ Ci in 30-40 μ l saline was injected percutaneously into the right testis of the rat with a gas-tight Hamilton syringe. A NaI crystal detector (Frisvick-Hoeftler 421 A) was used with 10 mm cut collector (Frisvick-Hoeftler 49 A scaler) and recording was made on Hewlett-Packard 717 A strip chart recorder. After background subtraction the recordings were plotted on semilogarithmic paper and the calculated 1/2 for the washout curve was introduced into the following formula, given by Kety (1951) in order to obtain blood flow.

$$F = \frac{\ln K \cdot 100}{1/2}$$

In particular coefficient, K, used was 0.85 (Selchell *et al.* 1966).

Methodological Aspects of Testicular Blood Flow Measurements in Rats

By

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Abstract

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Three techniques for the measurement of testicular blood flow in anesthetized adult rats were compared. The measurement of testicular venous outflow yielded values more than 3 times lower than those obtained with Xe-133 clearance and radioactive microsphere techniques due to the surgical procedures involving spermatic venous cannulation. There was an agreement between flow values obtained with Xe-133 clearance (17.8 ± 3.5 ml/100 g min) and radioactive microsphere technique (19.9 ± 3.5 ml/100 g min). A homogeneous distribution of microspheres in different segments of the testis indicates that Xe-133 clearance is a reliable technique for testicular blood flow measurements. However, for some experimental purposes the radioactive microsphere technique is more versatile than Xe-133 clearance because of its capacity of measuring multiple organ flows simultaneously.

Blood is the major route for the transport of androgens from the testes to the rest of the body (Setchell 1970). Since perfusion rate is of paramount importance for testosterone secretion (Eik-Nes 1964) many factors influencing the testicular blood flow may affect testicular hormone secretion. In studies of testicular endocrine function it is therefore important to measure circulatory variations in an accurate way.

Many techniques have been used to measure testicular blood flow in different species, such as direct measurement of venous outflow, Xenon-133 clearance, indicator fractionation (Setchell 1970), and, recently by use of a miniature friction flowmeter (Jaffe and Free 1976). The results reported are approximately of the same magnitude but information is lacking regarding the prerequisites for the use of a particular method in relation to other techniques. For instance, direct techniques involving venous cannulation and the use of flowmeters may disturb testicular blood flow in a way similar to that reported for the ovary (Jansson and Selstam 1975). Also, indirect techniques, like, for instance, indicator fractionation (Jansson 1958) may yield erroneous results due to an incomplete extraction of the indicator from the blood to the tissues, as was recently shown for abundant ovarian blood flow.

1. Testicular blood flow estimated by different methods in anesthetized adult rats.

of	No.	Testicular blood flow (ml/100 g min)
1. venous cannulation	15	5.9 ± 4.3
2. ¹³³ Xe clearance	25	17.8 ± 3.5
3. active microspheres	25	19.9 ± 5.5^a

a are given as mean \pm S.D.

as arterial pressure during measurements: 96 ± 25 mmHg.

right testis, epididymis and fat along the vascular pedicle were dissected and counted for radioactivity by the spermatic venous blood. The number of microspheres in the blood, divided by the number tested, was taken as an estimate of the degree of arterio-venous shunting of spheres.

testes, from rats infused with microspheres, are cut with sharp scalpel, in 4 segments from the head to the distal pole. The segments thus obtained contained different proportions of centrally and laterally located testicular tissue. Each segment was then counted separately for 5 min and the number spheres per mg testicular tissue was calculated.

Results

Comparison between different techniques (Table I)

Venous measurement. The testicular blood flow was 5.9 ± 4.3 ml/100 g min (mean \pm S.D.). In this preparation, $46.2 \pm 15.8\%$ (mean \pm S.D.) of the total venous outflow in the right testis represented testicular blood flow the rest originating from the epididymis, fat and connective tissue along the vascular pedicle.

Xenon-133 clearance. The blood flow in the right testis estimated by this method was 17.8 ± 3.5 ml/100 g min (mean \pm S.D.). Fig. 2 shows a typical clearance curve for Xenon-133 injected percutaneously into the testis. A stable linear curve was obtained after 3–4 min and lasted at least for 7–8 min.

Radioactive microspheres. Testicular blood flow as determined by radioactive microspheres was 19.9 ± 5.5 ml/100 g min (mean \pm S.D.). Blood flow values for the epididymis, ventral prostate and kidneys were 1.6 ± 4.22 , 37.6 ± 18.7 and 431.6 ± 139.7 ml/100 g min respectively (mean \pm S.D.).

Since it is known (Buckberg *et al.* 1971) that the variability of distribution of spheres to

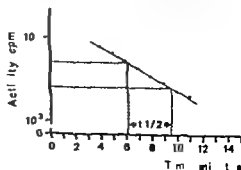


Fig. 2. A typical clearance curve for Xenon-133 injected percutaneously into the right testis of an adult rat.

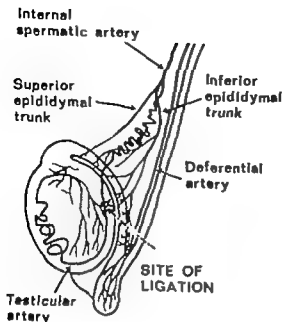


Fig. 1 Schematic illustration of the arterial to the testis and epididymis in the rat. The ligation of the testicular artery during the measurements is indicated in the figure.

Radioactive microspheres. Radioactive "carbonized" microspheres labelled with yttrium 89 and scandium 46 (^{89}Sc) and with a diameter of $15 \pm 5 \mu\text{m}$ were obtained from 3M Co. (St. Paul, U.S.A.) suspended in 20% (w/v) dextran. The initial specific activity of ^{89}Yb was 10.07 mCi/g and ^{89}Sc was 8.63 mCi/g . A drop of detergent was added to inhibit aggregation of spheres. Arterial spheres were transferred to a glass chamber holding a volume of 0.9 ml. The design of the chamber and handling of it were described in detail by Rudolph and Heymann (1967).

In 25 rats, catheters (PE 50) were inserted into the right brachial artery, the tail artery and into the ventricle or the ascending aorta via the right or left common carotid artery. The catheters are fitted with a solution of heparin and saline (1:10). The right brachial arterial catheter was connected to a St. P 23 AC transducer connected to a Grass Model 7 Polygraph. The glass chamber containing the spheres in dextran was vigorously agitated using a high-frequency mechanical stirrer and the sphere was flushed into the heart or ascending aorta for 30 s with 1 ml saline. 15 s before, during, and for 15 s after termination of the infusion of microspheres, blood was withdrawn at a constant rate (0.8 ml/min) from the tail arterial catheter. This reference sample was withdrawn into a 2 ml disposable plastic syringe attached to a pump adapted for aspiration on each rat. The first using ^{89}Yb labelled spheres, under conditions. A second injection was made in 13 of the rats, using ^{89}Sc labelled spheres, after lowering blood pressure by giving an additional 1.5 ml injection of barbiturate. After the second injection of ^{89}Sc "reference sample" the testes and some other organs and tissues were measured in a automatic scintillation counter (Packard Auto-Gamma). Each sample was counted for 3 min. By counting standard of known number of microspheres from the actual batches, the number of spheres in different organs and tissues was calculated. Blood flow were then calculated as follows (Rudolph and Heymann 1967):

$$F = \frac{Q_{\text{ref}}}{N_{\text{ref}}} \frac{N_{\text{organ}}}{N_{\text{ref}}}$$

where Q_{ref} = rate of withdrawal of the reference sample, N_{organ} = number of spheres present in the organ and N_{ref} = number of spheres present in the reference sample.

In order to obtain good accuracy with this technique blood flow values were not accepted when the number of spheres in the organ to be studied exceeded 400 (Buckberg *et al.* 1971).

Another 15 rats were laparotomized and the right internal spermatic vein was cannulated, using a PE catheter at least 5 mm from the entry into the caudal vein. Blood draining the testis, the epididymis, and fat and connective tissue along the vessels of the testis, was collected into small glass tubes for 5 min starting 2.5 min before an intracardiac infusion of Yb-labelled microspheres. After 15 min the rats

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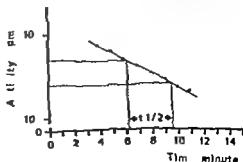


Fig. 2. A typical clearance curve for Xenon-133 injected percutaneously into the right testis of an adult rat.

TABLE II Distribution of 15 μ m radioactive microspheres in different segments of 39 rat testes.

Segment	Number of spheres per mg of wet tissue wt
A	1.8 ± 0.3
B	1.9 ± 0.3
C	1.7 ± 0.2
D	1.7 ± 0.2

Values given are means \pm S.E. Analysis of variance gave no significant differences in sphere distribution in different testicular segments.

any organ approximately follows a Poisson distribution accuracy of flow measured the 95% level could be calculated as follows

Accuracy (%) = $\frac{1.96}{X} \times 100$ where X is the number of spheres in the organ to be measured

In the present study the mean accuracy was 3.9% for the testes, 8.6% for the epididymis, 8.0% for the ventral prostate and 0.9% for the kidneys. A paired comparison between flow per unit of weight in the right and the left kidney gave no statistically significant differences according to Wilcoxon's paired t test based on range (Siegel 1956). The same results were obtained for the right and left testis, and the right and left epididymis. Spearman correlation coefficient r (Siegel 1956) for testicular and epididymal blood flow was 0.95 and was significant ($p < 0.01$). Blood pressure and heart rate were stable during the measurement of basal blood flows. Blood flow of the vascular pedicle, containing mostly testis, was 3.2 ± 0.3 ml/100 g min and blood flow of the lumbar muscle was 5.0 ± 0.8 ml/100 g min. These values are, however, uncertain since the number of spheres in the tissues was 400 on several occasions.

Distribution of blood flow within the testis

The number of spheres per mg of wet tissue in the 4 segments of the testis is presented in Table II. No statistically significant difference in distribution of spheres to different testicular segments was obtained by analysis of variance.

Arterio-venous shunting

The passage of radioactive microspheres from the testis, epididymis and tissues along the vascular pedicle to spermatic venous blood was 0.9 ± 0.4 (Table III).

Relationship between arterial pressure and testicular blood flow during sodium pentobarbitone anesthesia

In 13 of the non-laparotomized rats, an additional i.v. dose of sodium pentobarbitone was given immediately after the first blood flow measurement using Yb-169. Five to 10 min after the first measurement the second one was performed with Sc-46. The change in blood pressure was -12.9 ± 8.8 mmHg of the initial value, and the corresponding change in testicular blood flow was -12.6 ± 13.1 ml/100 g min. The correlation between arterial pressure and testicular blood flow was -0.8 . The testicular vascular resistance appeared to be increased by an additional dose of sodium pentobarbitone (12.7 ± 10.6 mmHg of initial value).

TABLE III. Passage of radioactive microspheres from testis, epididymis and tissues along vascular pedicle to spermatic venous blood.

Number of microspheres in testis, epididymis and pedicular fat (A)	Number of microspheres passed to spermatic venous blood (B)	A-V "shunting" (B/A) (%)
1,395 ± 515.3	15.5 ± 6.0	0.9 ± 0.4

Values are expressed as mean ± S.E.

Discussion

Testicular blood flow measurements with radioactive microspheres in the rat have not been reported elsewhere. The flow values of the present study are of the same magnitude as those reported for the rabbit by Neutze *et al.* (1968) using the same method, and are also in agreement with values reported by others using different methods (Seitchell 1966, 1970, Jaffe and Jaffe 1972).

When using the microsphere technique it is essential that certain basal requirements are fulfilled (Rudolph and Heymann 1972). The spheres should be evenly distributed in the arterial blood stream, they should be completely entrapped in the tissues under study and, finally, they should occur in sufficient numbers in the tissues to permit precision in the measurements. In the present study the lack of significant differences in blood flow between right and the left side in paired organs, indicates an even distribution of spheres and the fact of arterio-venous shunts larger than a diameter of 15 μ m indicates a complete arrest of spheres in the testis. Finally the number of spheres in the testis corresponded to a precision in the measurements of 2.5% ($\pm 95\%$ confidence level). Blood flow values of the present study of organs other than the testis were in agreement with those found by others with both microspheres and other methods. Bruce (1976) used microspheres in young male rats and reported somewhat lower values for renal blood flow (217 ± 44 ml/100 g/min) than obtained here for adult male rats. The values are, however, of the same magnitude. Seitchell *et al.* (1964) used the technique of Saperstein (1958) and reported values of epididymal blood flow very similar to those obtained here with microspheres. Anderson *et al.* (1967) described a method for measuring prostatic blood flow in dogs using Xenon-133 and reported values of 31.79 ml/100 g/min, which are in good agreement with those of the present study. Direct measurement of testicular blood flow by cannulation of the spermatic vein yielded values much lower than the other two methods used. However, when using radioactive microspheres in a preparation involving spermatic vein cannulation, the results are in excellent agreement with the present direct measurements (Damber and Jansson, to be published). This indicates that the direct measurement reflects true testicular blood flow but that the actual flow is reduced by the procedures involved in the measurement itself.

Direct measurement of testicular blood flow involves laparotomy which reduces blood flow in the testis (Damber and Jansson, to be published). Manipulation during the cannulation of the vein is also probably a factor reducing the testicular blood flow. As suggested

by Goding *et al* (1972), the flow can be reduced both by an increased venous pressure to the cannulation and by arterial spasm caused by manipulation.

The percutaneous Xenon-133 technique for measuring testicular blood flow described by Wax (1971). Using dogs and rats he obtained clearance curves similar to those obtained by the microsphere technique. Clearance techniques are most suitable for homogeneous tissues where a uniform flow can be expected (Kety 1951). Although there is a rapid decay in radioactivity during the first 1 to 2 min there is probably only one major component from the testis represented by the stable linear curve obtained after 3-4 min. The lack of linearity during the first part of measurement is probably caused by the escape of Xenon-133 through the tract of the needle (Wax 1971). However, Blombury and Waites (1968) interpreted the exponential clearance curves obtained by Krypton-85 clearance technique as a non-uniform perfusion of the testis. However, when using radioactive microspheres in the present study we were unable to find any differences in the distribution of spheres to different segments of the testis, indicating that blood flow throughout the organ is uniform and that the clearance technique should be applicable to measurements of testicular blood flow. This is supported by the fact that, in the present study, the Xenon clearance values were in good agreement with those of the microsphere technique.

Sodium pentobarbitone is one of the most widely used anesthetics for experimental animals. It is known to affect systemic and regional hemodynamics only slightly (Gardner *et al* 1976). In our experiments, barbiturate anesthesia resulted in stable blood pressure and heart rate. Free and Jaffe (1972) found similar values for testicular blood flow in conscious rats as compared to barbiturate anesthetized ones. This indicates that testicular blood flow values obtained during barbiturate anesthesia represent physiological flows, but the fall in blood pressure during anesthesia may be accompanied by a decrease in vascular resistance. In the present study an additional injection of barbiturate to the rats resulted in variable effects on blood pressure and testicular flow and inconsistency on vascular resistance. Therefore, it is very difficult to make any conclusions about the flow relationship for the testis with this design. However, in most cases the testicular flow was reduced when blood pressure decreased.

In summary, the results of the present investigation indicate that 1) the operative procedure associated with cannulation of the spermatic vein causes a significant decrease of testicular blood flow which is of importance to consider when evaluating testicular androgen production rate in studies involving analyses of spermatic vein blood, 2) the percutaneous Xenon-133 clearance technique and the microsphere technique are both useful for studies of testicular blood flow regulation in the rat testis, 3) the distribution of flow to different segments of the testis is uniform.

The particular aim of a study involving measurements of testicular blood flow is often considered when choosing between Xenon-133 clearance and microsphere technique. The simplicity, economy and suitability of Xenon-133 clearance for "chronic" experiments on certain occasions, be outweighed by the microsphere method because of its ability to measure several organ blood flows simultaneously yielding an extensive picture of the hemodynamic situation. The latter technique may, for instance, be the one of choice when trying to clarify whether a vascular effect of an agent is specific to the testis or not.

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C-fibres had little, if any physiological role to play. Thorén, Saum and Brown (1977) and *in vitro* and Thorén and Jones (1977) in the rabbit *in vivo* have studied the characteristics of aortic C-fibres in greater detail and found them to have higher thresholds and discharge frequencies than the medullated fibres. The C-fibres were thought to be only anti-hypertensive in nature (Thorén and Jones 1977).

reflex effects of aortic baroreceptor C-fibres have also been studied. Thus electrical stimulation of these non-medullated afferents in the aortic and sinus nerves can evoke power pressor reflexes in cats (Douglas and Ritchie 1956, Douglas, Ritchie and Schaumann) and rabbits (Kardon, Peterson and Bishop 1975) with different efferent patterns from medullated fibres (Kardon *et al.* 1975).

few of this work has studied some characteristics of C-fibres arising from the aortic arch in rabbits with chronic renovascular hypertension and compared them with medullated fibres arising from the same site.

Methods

1 hypertension was induced in 6 rabbits by either polyethylene wrapping of the left kidney (1 expt) or clipping of the left renal artery (3 expts) using silver clips of 0.7 or 0.8 mm internal diameter according to techniques of Rosero *et al.* (1973). In all animals the contralateral kidney was removed 10 days later. Another 14 rabbits were operated upon (3 polyethylene wrapping and 9 renal artery clipping) could not be included in the study because of either operative or postoperative deaths (7 rabbits) or lowest degree of hypertension (mean arterial blood pressure (MABP) less than 125 mmHg).

All rabbits blood pressures as checked pre-operatively when the animals were fully conscious and dated, by means of direct needle puncture of the ear artery. Each had been anesthetized with few of subcutaneous local anesthetic. Blood pressure as determined using the same method 14 or 20 days thereafter and finally one or two days before the acute expt. was carried out.

On the day of the acute expt. the rabbits were preanesthetized with diazepam (Valium 5 mg p) and then with sodium pentobarbital (30 mg/kg). A tracheal cannula was inserted, the animal was fixed with gallamine triethiodide (10 mg/kg) put on intermittent positive pressure ventilation. Small doses of anaesthetic or gallamine were given as required.

A femoral artery was cannulated and the cannula passed up to the arch of the aorta for measurement of aortic blood pressure. A femoral vein was cannulated for administration of fluid and drugs. The pH, P_aO_2 and PO_2 were monitored and the respiratory pump adjusted accordingly. Small doses of $NaHCO_3$ (20 ml) were given as required to correct any developing acidosis.

Incisions were made in the neck as made and the vagus and aortic nerves on the right side sectioned. The left vagus was also sectioned and the left aortic nerve dissected free for a distance of at least 3 cm. Allow adequate measurements of conduction velocity to be made. A pool was made from surrounding muscle and filled with paraffin oil. The left aortic nerve was placed on small plates and the nerve sheath and under binocular dissection microscope. Two filaments are teased free and placed on bipolar isolated silver electrode and connected to bipolar amplifier. The output from each was displayed on a oscilloscope (Tektronix 502) and on an ultraviolet light (UV) recorder (ABEM 3631). In addition, output from the amplifier was also led to loudspeaker and to ratemeter (time constant 0.4 s). The ratemeter had discriminator so that could count either all spikes exceeding pre-determined level or spikes with amplitudes lying between two pre-set levels. The output from the ratemeter was recorded on Grass polygraph Model 7D. It is as possible to check the accuracy of the ratemeter by direct counting spikes in the UV recordings and thus as done frequently throughout the expts.

The conduction velocities of both the C-fibres and the medullated fibres were calculated from the time delay between the application of an electrical stimulus to the aortic nerve and the appearance of the evoked action potential and from the distance between the stimulating and recording electrodes. In few cases the total time between stimulus applied directly to the receptor in the aortic arch and the recording electrode was measured.

C-fibre unit
the

is as obtained by recording from the filament during electrical thorax. All filaments showing C-fibre activity were further

Characteristics of Aortic Baroreceptors with Non-medullated Afferents Arising from the Aortic Arch of Rabbits with Chronic Renovascular Hypertension

By

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Abstract

JONES, J V and P N THORÉN *Characteristics of aortic baroreceptors with non-medullated afferents arising from the aortic arch of rabbits with chronic renovascular hypertension*. Acta physiol. scand. 1977 101 286-293

The characteristics of 47 non-medullated and 54 medullated *f* fibres arising from the aortic arches of 6 hypertensive rabbits have been investigated. The threshold for activation of the aortic C-fibres lay between 1 and 190 (mean 122) mmHg. The threshold for activation of 54 medullated aortic baroreceptors in the same animals was from 70 to 140 (mean 109) mmHg. At the awake mean arterial blood pressure (MABP) of these hypertensive rabbits (143 mmHg) all the medullated *f* fibres were active as were 78% of the C-fibres. In contrast in a group of normotensive animals 91% of the medullated and only 78% of the non-medullated *f* fibres were active. Pressure response curves were constructed for 19 C-fibres and 12 medullated *f* fibres from the hypertensive animals. At a MABP of 110 mmHg the firing in the non-medullated *f* fibres was 21 Hz as compared with 19.0 Hz in the medullated *f* fibres and the awake MABP mean firing in 9 Hz in the non-medullated fibres and 48.0 Hz in the medullated *f* fibres. The firing at awake MABP as considered indicate that although the aortic C-fibres are reset in chronic hypertension, they are reset less than the medullated aortic baroreceptors from the same animal. It is suggested that arterial baroreceptor C-fibres may have an important role in the tonic control of the circulation in hypertension.

There have been many studies over the years of the characteristics of the large myelinated fibres arising from the aortic arch and carotid sinuses in a variety of animals and even in man (Kirchheim 1976). Only recently have similar studies (Fidone and Sato 1969) begun to be performed on the small unmyelinated fibres arising from the baroreceptor region. These authors described the existence of baroreceptor C fibres although Landgren (1952) and Landgren, Neil and Zotterman (1952), many years earlier had described baroreceptor afferents in the sinus nerve with low signal to noise ratios which presumably were C-fibres. More recently Coleridge *et al* (1973) recorded from aortic C-fibres in the vagus nerve of the dog and described them as having very high thresholds. Coleridge *et al* thought that

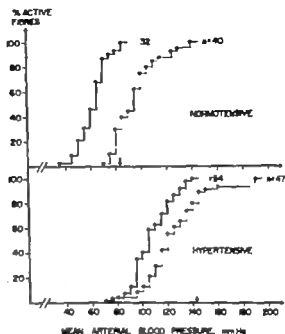


Fig. 2. The bottom panel summarizes the data shown in Fig. 1. The percentage of medullated and non-medullated fibres which have reached threshold, is plotted against the MABP. The upper panel shows, for comparison, data obtained in a similar way from 15 normotensive rabbits (Thorén and Jones 1977).

from very high threshold baroreceptors as have been described using an *in vitro* arch preparation in normotensive rats (Thorén *et al.* 1977).

The mean arterial blood pressure level at which the C-fibres either started firing or at which sporadic afferent traffic became synchronous with the cardiac cycle is shown for each fibre in each animal in Fig. 1. Similarly the threshold values for the medullated fibres are shown in Fig. 1. All of the medullated and 37 of the C-fibres have threshold values lower than the awake blood pressure of the animal, *i.e.* the great majority of the C-fibres would have been firing under normal circumstances in these hypertensive animals. The mean threshold for the medullated fibres is 109 ± 2 mmHg while that for the C-fibres is 122 ± 4 mmHg. There is considerable overlapping in the thresholds of the medullated and the non-medullated fibres, which was not the case in the normotensive group (Fig. 2).

The combined data on the thresholds for the medullated and the non-medullated fibres are shown in Fig. 2. For comparison the threshold data obtained in a similar way in normotensive rabbits (Thorén and Jones 1977) are shown in the upper part of the figure. At the awake mean blood pressure of the normotensive group 29 out of 32 (91%) medullated and 11 out of 40 (28%) of the C-fibres are firing. In the hypertensive group the equivalent figures are 34 out of 34 (100%) medullated fibres firing as are 37 out of 47 (78%) C-fibres. Although the C-fibre receptors are clearly reset in the hypertensive animals they have not reset to the same extent as the receptors with medullated fibres. The differences in blood pressure when 50% of both fibre groups have reached threshold are 30 mmHg for the normotensive animals and only 15 mmHg for the hypertensive rabbits (Fig. 2).

The receptor response to lowering the aortic arch pressure by caval vein occlusion and to raising the pressure by descending aortic occlusion was determined for 19 C-fibres

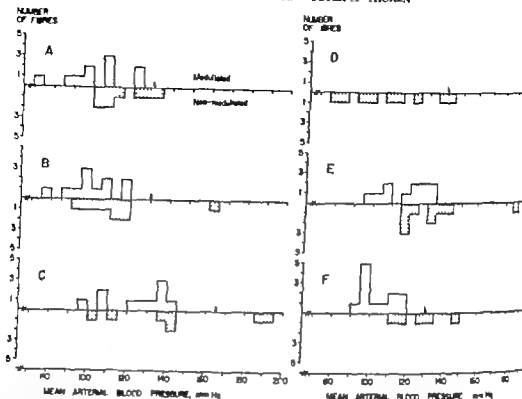


Fig. 1 The thresholds for activation of 54 medullated and 47 non-medullated fibres are shown. Each animal is plotted separately. The arrows indicate the awake mean arterial blood pressure in the individual animal.

dissected until only few fibre or in most cases, single fibre preparations remained. All such fibres were then tested by a brief occlusion of the descending thoracic aorta.

The receptors were then localized to the aortic arch region by their lack of response to brief ascending aortic occlusion and by their response to descending aortic occlusion. In some cases they were localized more accurately by gentle probing with a fine probe in which case they were found to be distributed throughout the aortic arch. A similar distribution was previously noted in normotensive rabbits (Thorén and Jones 1977) where the great majority of receptors had been localized by gentle probing.

Changes in aortic pressure were produced by occlusion of either inferior vena cava or descending thoracic aorta and the changes in receptor activity during such manoeuvres recorded.

Results

The awake mean blood pressure of the 6 rabbits prior to induction of hypertension was 85 ± 4 mmHg (mean \pm S.E.). At the time of study 6–9 weeks later the mean blood pressure was 143 ± 6 mm (for individual values see Fig. 1). The results described below are based on recordings from 47 C-fibres and 54 medullated fibres from the left aortic nerve. The conduction velocities for the C-fibres varied from 0.4 to 1.7 m/s (mean \pm S.E. 0.91 ± 0.4 m/s).

All receptors studied were localized to the aortic arch region by their response to occlusion of the descending aorta and the lack of response or decrease in activity when the ascending aorta was clamped. As in the normotensive rabbit (Thorén and Jones 1977) the aortic nerve appeared to contain C-fibres from endings that could be localized to the heart (confirmed in many cases by mechanical probing of the myocardium) and C-fibres to which a function could not be ascribed as they did not respond to the maximum pressure change attainable with this preparation (range 175–220 mmHg). It is possible that some of these

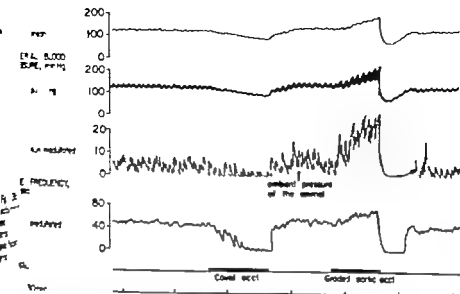


Fig. 2. Effect of graded caval occlusion and aortic occlusion on aortic blood pressure and spike frequency in one medullated and one non-medullated aortic baroreceptor afferent in the same filament. The medullated afferent has somewhat higher threshold and lower discharge rate than the medullated afferent. The mean awake blood pressure (128 mmHg) for this hypertensive rabbit is indicated by an arrow.

in the normotensive rabbit (Thoren and Jones 1977) were reached first when the pressure was increased above the normal resting value, whereas in the hypertensive rabbit the thresholds for the C-fibre receptors were largely obtained by lowering blood pressure. One question is then whether hysteresis (Angell James 1973) can explain some of the differences between the two groups of animals. This is, however not the case for two reasons.

First, in many hypertensive animals the thresholds were also obtained first during acute rise in blood pressure, because the aortic blood pressure of the anesthetized hypertensive rabbit is often lower than the awake blood pressure of the same animal.

Furthermore hysteresis is manifested as a higher threshold when the pressure is lowered than when the pressure is raised and, therefore, if hysteresis were of importance for the explanation of the present results it should tend to cause too high threshold value of the aortic C-fibres in the hypertensive rabbit and not as shown in Fig. 2 less prominent resetting of the aortic C-fibres.

The receptors were located to the aortic arch region according to their response to occlusion of descending aorta and the lack of response to occlusion of ascending aorta. In our study on the normotensive rabbits (Thoren and Jones 1977) this method of localization was adequate to locate the fibres to the aortic arch region.

Receptor characteristics

The most significant finding in this study is that even although the aortic C-fibres are reset during chronic hypertension they are clearly less reset than are the aortic medullated fibres

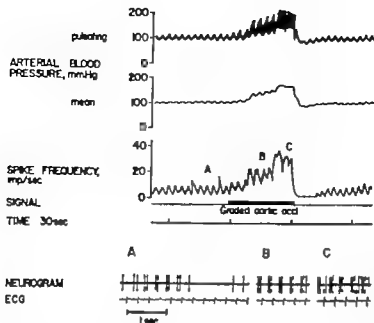


Fig. 3 Effect of a graded aortic occlusion on aortic blood pressure and spike frequency in a single aortic C-fibre. The lower middle spike frequency recordings correspond to the neurograms below.

and 12 medullated fibres. Fig. 3 shows the relationship between the discharge frequency and the mean arterial blood pressure in one of these C-fibres during graded aortic occlusion.

Fig. 4 shows the simultaneous discharge rate in one medullated and one C-fibre in the same filament during graded aortic occlusion. During the manoeuvres the different variables were recorded on a Philips analog 7 tape recorder so that they could be replayed and the spike counter first set to record the C fibre activity and then reset to record the activity of the medullated fibre. The C fibre has a higher threshold than the medullated fibre and smaller increase in discharge frequency with increasing pressure.

The combined data describing the blood pressure discharge relation for the 19 medullated and the 12 medullated fibres are shown in Fig. 5. This figure, of course, represents only C-fibres in which it was possible to obtain pressure response curves. The high threshold fibres (Thorén *et al* 1977, Thorén and Jones 1977) may have different response frequencies from those C fibres observed here.

Total conduction time was obtained for 3 receptors and the firing in relation to the cardiac cycle could then be determined. Activation of the receptors were then found to occur in early systole in phase with activation of the medullated fibres. Thus they are similar to the C-fibre traffic in normotensive rabbits (Thorén and Jones 1977).

Discussion

Further evidence for the presence of C-fibres arising from the aortic arch was obtained from this study. Such fibres have recently been described in normotensive animals (Ficker and Saito 1969, Coleridge *et al* 1973, Thorén *et al* 1977, Thorén and Jones 1977).

Experimental methods In this study the thresholds and pressure response curves of medullated and non-medullated aortic baroreceptors were compared in normotensive and hypertensive rabbit. The results in Fig. 2 suggest that the threshold values for the majority of the

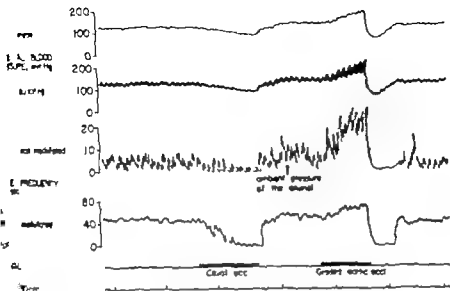


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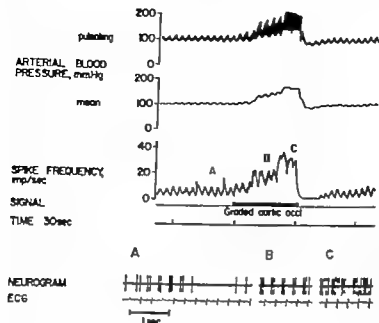


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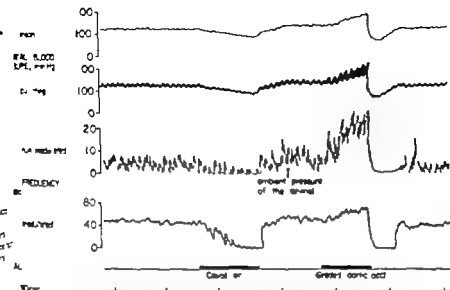


Fig. 2. Effect of graded caval vein occlusion and aortic occlusion on aortic blood pressure and spike frequency in a normotensive and one non-medullated aortic baroreceptor afferent in the same filament. The non-medullated afferent has a somewhat higher threshold and lower discharge rate than the medullated afferent. The mean awake blood pressure (128 mmHg) for this hypertensive rabbit is indicated by an arrow.

plots in the normotensive rabbit (Thorén and Jones 1977) were reached first when the blood pressure was increased above the normal resting value, whereas in the hypertensive rabbit the thresholds for the C-fibre receptors were largely obtained by lowering blood pressure. One question is then whether hysteresis (Angeli James 1973) can explain some of the differences between the two groups of animals. This is, however, not the case for two

first, in many hypertensive animals the thresholds were also obtained first during acute rises in blood pressure, because the aortic blood pressure of the anesthetized hypertensive rabbit was often lower than the awake blood pressure of the same animal.

Furthermore hysteresis is manifested as a higher threshold when the pressure is lowered compared when the pressure is raised and, therefore, if hysteresis were of importance for the explanation of the present results it should tend to cause too high threshold value of the aortic C-fibres in the hypertensive rabbit and not as shown in Fig. 2 a less prominent resetting of the aortic C-fibres.

The receptors were located to the aortic arch region according to their response to occlusion of descending aorta and the lack of response to occlusion of ascending aorta. In our previous study on the normotensive rabbits (Thorén and Jones 1977) this method of localization was adequate to locate the fibres to the aortic arch region.

Further characteristics

The most significant finding in this study is that even although the aortic C-fibres are reset during chronic hypertension they are clearly less reset than are the aortic medullated fibres

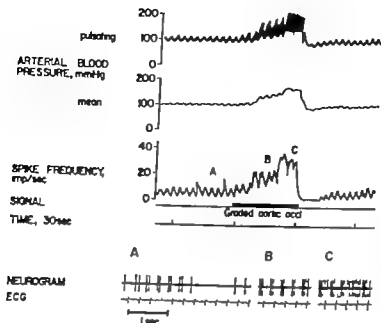


Fig. 3. Effect of a graded aortic occlusion on aortic blood pressure and spike frequency in a single aortic C-fibre. The letters A, B and C refer to the spike frequency recording and correspond to the neurogram below.

and 12 medullated fibres. Fig. 3 shows the relationship between the discharge frequency and the mean arterial blood pressure in one of these C-fibres during graded aortic occlusion.

Fig. 4 shows the simultaneous discharge rate in one medullated and one C-fibre in the same filament during graded aortic occlusion. During the manoeuvres the different variables were recorded on a Philips analog 7 tape recorder so that they could be replayed and the spike counter first set to record the C-fibre activity and then reset to record the activity of the medullated fibre. The C fibre has a higher threshold than the medullated fibre and smaller increase in discharge frequency with increasing pressure.

The combined data describing the blood pressure discharge relation for the 19 non-medullated and the 12 medullated fibres are shown in Fig. 5. This figure, of course, represents only C fibres in which it was possible to obtain pressure response curves. The high threshold fibres (Thorén *et al.* 1977; Thorén and Jones 1977) may have different response frequencies from those C-fibres observed here.

Total conduction time was obtained for 3 receptors and the firing in relation to the cardiac cycle could then be determined. Activation of the receptors were then found to occur in early systole in phase with activation of the medullated fibres. Thus they are similar to the C-fibre traffic in normotensive rabbits (Thorén and Jones 1977).

Discussion

Further evidence for the presence of C fibres arising from the aortic arch was obtained from this study. Such fibres have recently been described in normotensive animals (Ficker and Sato 1969; Coleridge *et al.* 1973; Thorén *et al.* 1977; Thorén and Jones 1977).

Experimental methods. In this study the thresholds and pressure response curves of medullated and non-medullated aortic baroreceptors were compared in normotensive and hypertensive rabbit. The results in Fig. 2 suggest that the threshold values for the majority of the

role of baroreceptor C-fibres in cardiovascular control in hypertension

observation that there is an increased firing in the C-fibres at pressures equal to awake pressure levels makes it likely that the C-fibres in hypertension exert a tonic influence on the vasomotor centre while in the normotensive animal it appears that they are largely quiescent in nature. For this reason, in hypertensive animals, they may help to permit for blood pressure reductions such as may result e.g. from hemorrhage, while in normotensive animals they are less effective in this respect since the firing in the C-fibres is sparse under normal conditions.

In conclusion, therefore, the C-fibres arising from the aortic arch of hypertensive animals are not just as the modulated receptors. However the resetting appears to be less complete. Consequently baroreceptor C-fibres may be of major importance for the tonic, homeostatic control in the hypertensive animal. Reflex studies are required to determine the degree of truth in this assertion.

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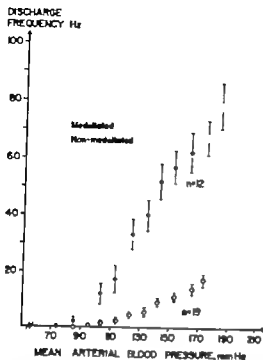


Fig. 3 Pressure response curves for 19 non-medullated and 12 medullated aortic baroreceptors in 6 hypertensive rabbits.

in the same animal. Thus when 50% of the aortic medullated and 50% of the non-medullated fibres are firing the differences in threshold were twice as great in the normotensive comparison with the hypertensive rabbits. Furthermore, in the hypertensive animals 21 of the aortic C-fibres are firing at ambient blood pressures in contrast to only 28% in a normotensive group of animals as shown by Thorén and Jones (1977). In addition the firing rate in the C-fibres was 1.1 Hz at the mean ambient pressure of the normotensives (Thorén and Jones 1977) while it was 9.0 Hz at the equivalent pressure in the hypertensives. Thus even under normal awake conditions there is a greatly increased impulse traffic from C-fibre receptors in the hypertensive animals compared to the normotensive controls, implying that they exert a more pronounced effect on higher vasomotor centres in the former. It is worth noting that also the mean traffic in the 12 medullated fibres examined in the hypertensive animals was higher at ambient pressure than the traffic in 12 medullated fibres in the normotensives (Thorén and Jones 1977). Thus the mean firing rate at awake pressures in the normotensives was 19 Hz in contrast to 48 Hz in the hypertensives.

Why do the C-fibres not reset as much as the medullated fibres?

Resetting of the medullated baroreceptors has been postulated to be due to either altered distensibility of the receptor area (Aars 1968) to degeneration of the baroreceptor sensory endings (Abraham 1967; Hilgenberg 1967) or to a combination of the two (Angel 1967; Jones 1973). If altered distensibility of the aortic arch is responsible for resetting, and it is likely that this is at least partly the case since reversal of aortic baroreceptor resetting has been shown to occur within a matter of hours after abolition of the hypertension (Salpekar and Krieger 1973), then the different degree of resetting seen with the C-fibre receptors implies that they are linked up to the wall of the aorta in a different manner or at a different site from the medullated fibre receptors.

Effect of the catecholamine with the β -receptor The sequence of events leading from the interaction between the β -adrenoceptor and an agonist to a response has been particularly well characterized in the adipose tissue (cf Fain 1973). We have therefore used this system to study the effects of acidosis *in vitro* and *in vivo*. We recently showed that acidosis inhibits cyclic AMP formation in isolated rat fat cells (Fredholm and Hjemdahl 1976). However lipolysis at decreased pH did not reach control levels even if fat cyclic AMP increased to supranormal levels (Hjemdahl and Fredholm 1976a). This suggests that acidosis inhibits lipolysis also at a step subsequent to cyclic AMP formation.

It is possible that the antilipolytic effect of acidosis is mediated by an endogenous inhibitor of lipolysis. At present there are three main candidates for a role as endogenous inhibitor of lipolysis: adenosine (Schwabe *et al.* 1975), fatty acids (Rodbell 1965 Fain and Shepherd 1975) and the so-called feed-back regulator of Ho and Sutherland (1975). It is also possible that pH changes could induce changes in the cellular cation composition (Brown and Gooty 1963, Carvalho *et al.* 1963), which may influence lipolysis (Mossinger 1970).

The antilipolytic effect of acidosis mediated over the accumulation of an endogenous factor or a change in ionic composition may be considered an "indirect" effect of acidosis. The purpose of the present report is to examine whether a decrease in pH is antilipolytic *via* such an indirect mechanism of action or whether the inhibitory effect can best be explained on the basis of a direct effect of the hydrogen ion. We have therefore examined the influence of modifications of the incubation media. Furthermore, the effect of decreased pH on lipolysis in "perfused" fat cells, as described by Allen and co-workers (1973, 1976) is examined. The latter technique has two advantages: firstly it reduces the influence of extracellular material(s) in the medium surrounding the fat cells and secondly it allows a kinetic analysis of activation and inhibition of lipolysis.

Materials and Methods

Isolation of fat pads from 3-5 Sprague-Dawley rats (Aurum-stress), weighing 180-220 g, were pooled and each experimental fat cell preparation is prepared as described by Rodbell (1964), using incubation with 3 mg/ml of bacterial collagenase (Worthington) for 40-60 min.

Isolation experiments The fat cells were counted in a Barker chamber and diluted to a final concentration of $50-70 \times 10^6$ cells/ml in Krebs-Ringer phosphate buffer containing, unless otherwise stated, 5.5 mM glucose, 1.8 mM CaCl_2 and 3 μM albumin (Bovine fraction V, Sigma, St. Louis). When the ionic composition of the buffer was adjusted to give a constant ionic strength. The cells were preincubated at 37°C, usually for 15 min, before the addition of lipolytic drugs.

Perfusion experiments The experiments with "perfused" fat cells were conducted essentially as described by Allen and co-workers (1973). Thus, 1 ml of packed fat cells are placed in a temperature (37°C) controlled, closed perfused chamber containing 2 ml buffer and were perfused at the rate of 2 ml/min, with Krebs-Ringer phosphate buffer of indicated pH containing 1.8 mM CaCl_2 , 5.5 mM glucose and 1 μM albumin. Lipolysis was stimulated by the infusion of noradrenaline (as the hydrochloride, Sigma, St. Louis), diluted buffer containing 20 $\mu\text{g/ml}$ ascorbic acid, via a side arm in the perfusion system. In some experiments 10^{-7} M-noradrenaline hydrochloride (7.5 $\mu\text{g/ml}$ mol New England Nuclear, Chicago) was added in order to determine the shape of the noradrenaline concentration profile. Our results in this respect agree completely with those of Allen *et al.* (1973, 1976).

Lipolysis Lipolysis was determined as glycerol production (in nmol/min) and in some experiments fatty acids were also determined by a titrimetric method (Trost *et al.* 1960). In the incubation experiments glycerol was measured exactly as described by Lowell and Tibbling (1966). In the perfusion experiments the lipid release was determined by a titrimetric method. Standard curves are therefore prepared using albumin-containing

Direct Antilipolytic Effect of Acidosis in Isolated Rat Adipocytes

By

PAUL HJEMDAHL and BERTIL B FREDHOLM

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Abstract

HJEMDAHL, P. and B. B. FREDHOLM. Direct antilipolytic effect of acidosis in isolated rat adipocytes. *Acta physiol. scand.* 1977 101 294-301.

The possibility that acidosis inhibits lipolysis indirectly by causing ionic shifts or by favouring the accumulation of an inhibitor has been tested in isolated fat cells. Lipolysis induced by 3 μ M noradrenaline (NA) was inhibited by 40-60% and that induced by 1 mM theophylline (THEO) by about 75%. Reducing the concentration of Ca^{++} or Mg^{++} did not alter the degree of inhibition. Reducing the H^+ -ion concentration enhanced the inhibitory effect of low pH on lipolysis induced by NA or NA + THEO, whereas cyclic AMP accumulation was uninfluenced. Omitting glucose from the incubation medium caused a slight enhancement of pH-induced inhibition of lipolysis (from 60 to 70%, $p < 0.01$). Reducing the concentration of albumin, which binds inhibitory substances such as FFA, reduced lipolysis more at normal than at reduced pH. At high FFA/albumin ratios (5 or above) lipolysis was similar at normal and reduced pH. The antilipolytic effect of decreased pH was equally pronounced in perfused fat cells, where inhibitory substances are not allowed to accumulate. Our results suggest that the antilipolytic effect of acidosis is mainly direct, due to the increase in H^+ -ion concentration. The inhibitory effect of acidosis on various responses to β -adrenoceptor stimulation may be caused by a decreased formation of cyclic AMP in turn caused directly by the decrease in pH.

Changes in pH influence the responses to catecholamines and to sympathetic nerve stimulation. There seems to be general agreement that acidosis counteracts those effects of catecholamines that are mediated over β -adrenoceptors. Thus it has been shown that adrenergic β -effects in the heart (Mitchell *et al.* 1972, Shaer 1974), bronchial (Blumenthal *et al.* 1969) and vascular (Wagner *et al.* 1975) smooth muscle are counteracted by decreased pH. Furthermore, metabolic effects of β -adrenergic stimulation are inhibited by acidosis (Poyart & Nahas 1966, Reynolds and Haugaard 1967).

It is unlikely that the inhibition of the catecholamine effect by reducing pH from 7.4 to 6.6 is due simply to a change in the degree of ionization of the amine (Reynolds & Haugaard 1967). It is also unlikely that the effect is specifically located to the catecholamine receptor, since lipolysis induced by ACTH is inhibited to a similar degree as that induced by noradrenaline (Wenkeová and Mosinger 1965, Poyart and Nahas 1968). Thus, the inhibition of adrenergic β -effects by acidosis is probably due to inhibition of a step beyond the

Interaction of the catecholamine with the β -receptor The sequence of events leading from interaction between the β -adrenoceptor and an agonist to a response has been particularly well characterized in the adipose tissue (*cf* Fain 1973). We have therefore used this to study the effects of acidosis *in vivo* and *in vitro*. We recently showed that acidosis inhibits cyclic AMP formation in isolated rat fat cells (Fredholm and Hjemdahl 1976). However, lipolysis at decreased pH did not reach control levels even if fat cyclic AMP increased to supramaximal levels (Hjemdahl and Fredholm 1976a). This suggests that acidosis inhibits lipolysis also as a step subsequent to cyclic AMP formation.

It is possible that the antilipolytic effect of acidosis is mediated by an endogenous inhibitor of lipolysis. At present there are three main candidates for a role as endogenous inhibitor of lipolysis: adenosine (Schwabe *et al* 1975), fatty acids (Rodbell 1965; Fain and Shepherd 1969) and the so-called feed-back regulator of Ho and Sutherland (1975). It is also possible that pH changes could induce changes in the cellular cation composition (Brown and Goettl 1963; Carvalho *et al* 1963), which may influence lipolysis (Mowbray 1970).

The antilipolytic effect of acidosis mediated over the accumulation of an endogenous inhibitor or a change in ionic composition may be considered an "indirect" effect of acidosis.

The purpose of the present report is to examine whether a decrease in pH is antilipolytic *in vitro* via such an indirect mechanism of action or whether the inhibitory effect can best be explained on the basis of a direct effect of the hydrogen ion. We have therefore examined the influence of modifications of the incubation media. Furthermore, the effect of decreased pH on lipolysis in "perfused" fat cells, as described by Allen and co-workers (1973, 1976) is examined. The latter technique has two advantages, firstly it reduces the influence of extracellular material in the medium surrounding the fat cells and secondly it allows a kinetic analysis of activation and inhibition of lipolysis.

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In some experiments in order to determine the shape of the noradrenaline concentration profile. Our results in this respect agree completely with those of Allen *et al* (1973, 1976).

Lipolysis. Lipolysis was determined as glycerol production (nmol/mg wet time) and in some experiments fatty acids were also determined by a titrimetric method (Trost *et al* 1960). In the incubation experiments, glycerol was measured exactly as described by Lissell and Tibbling (1966). In the perfusion experiments the initial decomposition step was omitted. Standard curves are therefore prepared using albumin-containing

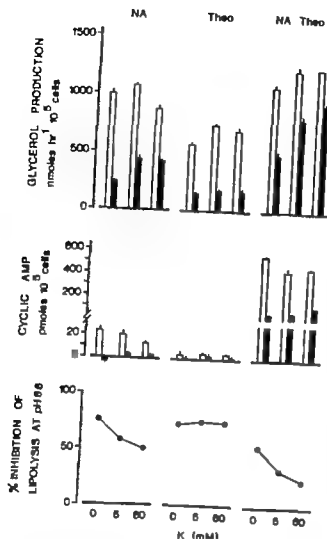


Fig. 1 Glycerol production (during 60 min) and net accumulation of cyclic AMP (30 min) in fat cells incubated with $3 \mu\text{M}$ noradrenaline (NA), 1 mM theophylline (Theo) or the combination of these drugs at pH 7.4 (open bars) and pH 6.6 (filled bars). The cells ($50\,000/\text{ml}$) were incubated in buffer containing either no, normal (5 mM) or 60 mM K⁺ with corresponding adjustment of the Na⁺ concentration. A preincubation time of 20–30 min was allowed before addition of a drug. The number of observations with each treatment is indicated along the columns denoting glycerol production. The lowest panel shows the percent inhibition produced by decreased pH. Note that it influences the antilipolytic effect of decreased pH in NA-stimulated fat cells but not in Theo-stimulated cells.

media in these expts. No significant interference was, however, detected. Cyclic AMP was determined by the competitive binding assay of Brown *et al.* (1977) as described previously (Fredholm and Hjermdahl 1976).

Results

Stimulated glycerol production and cyclic AMP formation was inhibited by actions as described earlier (Fredholm and Hjermdahl 1976, Hjermdahl and Fredholm 1976a). Thus, noradrenaline ($3 \mu\text{M}$)-stimulated lipolysis was inhibited by 50–60% at pH 6.6. Theophylline (1 mM)-stimulated lipolysis was inhibited by about 75% and lipolysis stimulated by the combination of these drugs by 20–30% (Fig. 1).

Effects of changes in the cationic composition The results from our expts, where the potassium concentration was either 0.5 or 60 mM are summarized in Fig. 1. Omission of K⁺ decreased lipolysis in all situations tested. In agreement with earlier findings (Mosinger 1970). In contrast, cyclic AMP accumulation was not significantly decreased when potassium was omitted. The inhibition of lipolysis by decreased pH was enhanced when potassium was omitted from the incubation medium, except when theophylline alone was used as the

(1) Inhibition of glycerol release and cyclic AMP accumulation in isolated rat fat cells by acidosis (pH 6.6) in the absence and in the presence of glucose (5.5 mM). The values are means \pm S.E. of quadruplicate incubations.

Glucose	Lipolysis at pH 6.6 (% of control)	cAMP accumulation at pH 6.6 (% of control)
0	26 \pm 1 33 \pm 2 p 0.05	36 \pm 13 22 \pm 5
0	31 \pm 2 42 \pm 2 p 0.01	44 \pm 17 30 \pm 8

clating agent. The inhibition due to decreased pH was significantly less pronounced ($p < 0.001$) in high K^+ -buffer when noradrenaline or noradrenaline + theophylline were used as stimulating agents.

A 10-fold increase in the magnesium ion concentration (from 1.2 to 12 mM) reduced adrenaline (3 μ M)-stimulated lipolysis by about 10%, but did not alter the cyclic AMP accumulation. The inhibition of lipolysis by decreased pH was unaffected by the change in magnesium concentration. By omission of Ca^{2+} and the addition of 1 mM EGTA noradrenaline (3 μ M)-stimulated lipolysis was inhibited by approximately 10% (590 ± 30 vs. 650 ± 30 nmol h $^{-1}$ 10^{-6} cells; $n = 5$). The antilipolytic effect of pH reduction was not affected by the omission of calcium ions (53% inhibition with Ca^{2+} vs. 50% inhibition without).

Effect of altered glucose and albumin concentration. Since it has been suggested that glucose counteracts the antilipolytic effect of acidosis (Poyart *et al.* 1967) experiments were carried out in the presence and absence of glucose. As seen in Table I our data confirm the previous observation, but the effect is minor. The inhibition of cyclic AMP accumulation was not affected by omitting glucose ($p = 0.02$, one-tailed t -test).

It has also been reported that excess albumin could reduce the antilipolytic effect of acidosis (Poyart *et al.* 1967). We could not confirm this finding. On the contrary as shown in Fig. 2, decreasing the pH to 6.6 produced more marked inhibition of noradrenaline (3 μ M)-stimulated lipolysis when the FFA/albumin-ratio was kept low by the addition of excess albumin. There was a significant inverse relationship ($r = -0.84$, $n = 18$) between the rate of lipolysis and the FFA/albumin ratio at a normal pH. At pH 6.6 the reduction in lipolysis with increasing the FFA/albumin ratio was significantly ($p = 0.01$ analysis of variance) less pronounced. This obviously suggests that inhibition of lipolysis by acidosis is not affected by increasing the FFA/albumin ratio are two independent processes.

Perfused fat cells. The inhibition of NA-stimulated lipolysis by acidosis was studied in 2 experiments with perfused fat cells (Table II). As shown previously in incubated fat cells (Hjorndahl and Hjorndahl 1976) the inhibition was more pronounced the lower the pH. The effect of acidosis was assessed by comparing the lipolytic effect of noradrenaline in two aliquots of fat cells, one perfused with buffer pH 7.4 one with buffer of a lower pH. The noradrenaline concentration in the medium rose and fell with a half-time of less

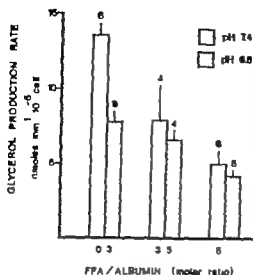


Fig. 2. The influence of FFA accumulation on the glycerol production rate in isolated fat cells (3000) incubated with 3 μ M noradrenaline at normal or reduced pH. The albumin concentration of the incubation buffer varied between 0.5 and 5 μ M. The molar ratio of FFA/albumin in incubates containing various FFA concentrations were determined at 40 and 80 min. Incubation with noradrenaline. The glycerol production rate during the 40 min periods of incubation (bars); these determinations is shown in incubates with (0-3), intermediate (3-5) or high (more than 5 FFA) albumin ratios. The number of observations (n) are indicated in the figure. Note that accumulation of FFA (i.e. a high FFA/albumin ratio) has a more pronounced inhibitory effect at normal than at decreased pH.

than 2 min both at pH 7.4 and pH 6.6 (not shown). Glycerol production rose much more slowly reaching a plateau after 15-25 min both at normal and reduced pH. After cessation of noradrenaline infusion the rate of glycerol production returned towards normal with a half time of 5-7 min at normal and reduced pH. These results are similar to those reported earlier for noradrenaline-stimulation in fat cells perfused with Krebs-Ringer bicarbonate buffer pH 7.4 without glucose (Allen *et al.* 1973, 1976).

In 3 other expts. the pH of the perfusing buffer was changed while the cells were exposed to noradrenaline. One expt. of this type is illustrated in Fig. 3. It is seen that the change in pH made the cells approach a new steady level of lipolysis, which was reached after 11 min.

Discussion

Acidosis inhibits lipolysis in 3 experimental situations: incubated fat cells, perfused cells and *in situ* perfused dog adipose tissue. These preparations should differ markedly with respect to the accumulation of inhibitory substances. As seen in Table III acidosis inhibits lipolysis to a similar degree in these different preparations. This may be due

TABLE II Net lipolytic responses to noradrenaline of fat cells (1 ml) perfused with bicarbonate buffer (pH 7.4 (control) and lower pH

pH	Noradrenaline μ M	Glycerol production (nmol min ⁻¹ ml cells ⁻¹)		inhibition
		Control	Low pH	
7.0	0.3	35	21	39
	1.0	45 \pm 9 ^a	30 \pm 4	31 \pm 9 ^a
7.0	10	38 \pm 10 ^b	20 \pm 6 ^b	42 \pm 15 ^b
7.0	1.0	7	1	56
6.8	1.0	45	15	67
6.6	1.0	23	4	81
6.6	1.0			

^a Mean \pm S.E. from 4 observations, 3 of which were of the type illustrated in Fig. 3.
^b Mean values \pm S.E. from 4 paired observations.

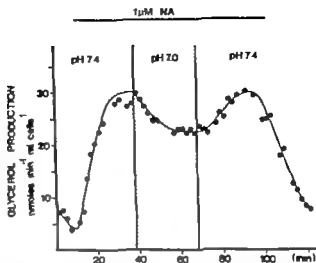


Fig. 3. Glycerol production rate in 3T3 fat cells (1 ml packed) perfused with saline buffer. Lipolysis was stimulated with 1 μ M NA during 90 min, as indicated at the figure. Note that the rate changes from one steady state to another approximately 10 min both after reduction of pH and change in pH after NA stimulation.

First argument against the effect of acidosis being mediated over an accumulating inhibitor. The second argument is derived from an examination of the kinetics in the perfused cell system. After noradrenaline infusion lipolysis returned towards basal rates with a half-life of 5-7 min irrespective of whether the cells were perfused with buffer at pH 7.0 or 6.6. This implies that acidosis does not change the rate-limiting step in the inhibition of lipolysis. Furthermore, the time necessary to go from one steady-state to other was the same whether the transition was from basal to stimulated at pH 7.4 or 6.6 or from stimulated pH 7.4 to stimulated pH 7.0 and vice versa (e.g. Fig. 3). These findings are not easily reconciled with an indirect mechanism of action of acidosis. Thirdly, we have examined more directly the importance of different possible mediators of the antilipolytic effect of acidosis.

Modifications of the calcium or magnesium ion concentrations in the medium had no effect on the antilipolytic effect of acidosis, even though some effect on the magnitude of the response to noradrenaline was seen. Changes in the distribution of these ions, which may occur during acidosis (Carvalho *et al.* 1963), therefore seem to have negligible influence on the antilipolytic effect of acidosis.

There is, however, evidence that K⁺ ions may somehow be involved in the inhibitory effect of acidosis. Decreased pH may cause a shift of potassium out of cells (Brown and Root 1963). We found that an increased potassium ion concentration counteracted the antilipolytic effect of acidosis when NA was used as the stimulating agent. K⁺ ions appear to influence a step beyond the formation of cyclic AMP since lipolysis was depressed by adding potassium, without significant effect on cyclic AMP. Furthermore, the effect of K⁺ on lipolysis was also seen when NA and theophylline were combined as lipolytic agents. Since this combination gives cyclic AMP levels in the fat cells that are supramaximal with respect to lipolysis (Hjerdahl and Fredholm 1976 a), this opens the possibility that the pronounced cyclic AMP-independent inhibition of lipolysis is caused by a decrease in the intracellular K⁺ ion concentration.

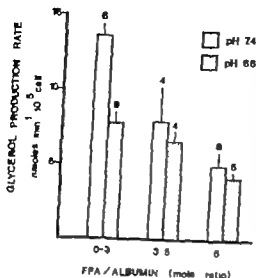


Fig. 2. The influence of FFA accumulation on glycerol production rate in isolated fat cells (90 min incubation with 3 μ M noradrenaline at normal reduced pH. The albumin concentration of the reaction buffer varied between 0.5 and 5 μ M. The ratio of FFA/albumin in incubates containing various concentrations were determined in 40 and 80 min incubation with noradrenaline. The glycerol production rate during the 40 min periods of incubation like these determinations is shown in incubates at (0-3), intermediate (3-5) or high (more than 5) albumin ratios. The number of observations are indicated in the figure. Note that accumulation FFA (*i.e.* a high FFA/albumin ratio) has pronounced inhibitory effect at normal than at decreased

than 2 min both at pH 7.4 and pH 6.6 (not shown). Glycerol production rose much more slowly reaching a plateau after 15-25 min both at normal and reduced pH. After cessation of noradrenaline infusion the rate of glycerol production returned towards normal in half time of 5-7 min at normal and reduced pH. These results are similar to those reported earlier for noradrenaline-stimulation in fat cells perfused with Krebs Ringer bicarbonate buffer pH 7.4 without glucose (Allen *et al.* 1973, 1976).

In 3 other expts. the pH of the perfusing buffer was changed while the cells were exposed to noradrenaline. One expt. of this type is illustrated in Fig. 3. It is seen that the change in pH made the cells approach a new steady level of lipolysis, which was reached after 11 min.

Discussion

Acidosis inhibits lipolysis in 3 experimental situations: incubated fat cells, perfused cells and *in situ* perfused dog adipose tissue. These preparations should differ markedly with respect to the accumulation of inhibitory substances. As seen in Table III acidosis inhibits lipolysis to a similar degree in these different preparations. This may be taken

TABLE II. Net lipolytic responses to noradrenaline of fat cells (1 ml) perfused with 2 ml buffer of pH 7.4 (control) and lower pH.

pH	Noradrenaline μ M	Glycerol production (moles mm ⁻¹ ml cells ⁻¹)			inhibition
		Control	Low pH		
7.0	0.3	35	1		19
7.0	1.0	45 \pm 9 ^a	30 \pm 4		31 \pm 9 ^a
7.0	10	38 \pm 10 ^b	20 \pm 6 ^b		42 \pm 15 ^b
6.8	1.0	27	12		56
6.6	1.0	45	15		67
6.6	1.0	23	4		81

^a Mean values \pm S.E. from 4 observations, 3 of which were of the type illustrated in Fig. 3.
^b Mean values \pm S.E. from 4 paired observations.

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TABLE III Inhibition of NA-induced lipolysis *in vitro* and *in vivo* by decreased pH

	pH 7.0 (% of response at pH 7.4)	Ref.
Isolated fat cells (3 μ M NA)	(45) ^a	b
Perfused fat cells (10 μ M NA)	4	Table D
<i>IV</i> NA infusion (0.35 μ g h ⁻¹ min ⁻¹)— constant flow perfused canine adipose tissue	56	
Sympathetic nerve stimulation (4 Hz)— free flow perfused canine adipose tissue	40	d

Since the pH is not stable during conventional incubation of fat cells this figure was obtained by interpolation.

^a Fredholm and Hjemdahl 1976

^b Hjemdahl and Fredholm 1976 b.

^d Hjemdahl 1976.

Adenosine is known to inhibit cyclic AMP accumulation in fat cells while lipolysis is inhibited to a minor degree, if at all (e.g. Hjemdahl and Fredholm 1976, Schwabe *et al.* 1975). Acidosis, on the other hand, inhibits lipolysis at least in parallel with inhibition of cyclic AMP accumulation (Fredholm and Hjemdahl 1976, Hjemdahl and Fredholm 1976). Thus, it is unlikely that the antilipolytic effect of acidosis is due to adenosine. The inhibitory effect of adenosine is, however, potentiated by a decreased pH (Hjemdahl and Fredholm 1976 a) and the two might act synergistically.

Both free fatty acids (Rodbell 1965) and the feed-back regulator of Ho and Sotthorn (1975) are strongly bound to and antagonized by albumin. Therefore our finding that the antilipolytic effect of acidosis was enhanced rather than depressed by increasing the albumin concentration in the medium (Fig. 2) argues against the opinion that either of these inhibitors mediate the effect of acidosis. Furthermore direct measurements of cell-associated fatty acids showed that their level was lower at reduced than at normal pH (Fredholm and Hjemdahl 1976). Thus, there is no good evidence that either of these proposed feedback inhibitors mediate the effect of acidosis.

We feel it is justified to conclude from these studies that the effect of acidosis on lipolysis is not to any major degree mediated over the accumulation of any endogenous inhibitor or to transcellular shifts of potassium, magnesium or calcium. This finding may be relevant for our understanding of how acidosis inhibits also other β -adrenoreceptor responses. In particular the possibility must be considered that acidosis causes an inhibition of cyclic AMP formation in most tissues, just as it does in fat cells and that this provides an explanation for the antagonism of β -adrenoreceptor responses thought to be mediated over cyclic AMP.

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It can give rise to erroneous estimations of the quaternary amines. Naturally those interfering factors that are recognized and understood can mostly be eliminated by appropriate means, but it is still uncertain whether other unpredicted factors are present or not. An important disadvantage of bioassay methods is that they are often very laborious and consuming.

In recent years great progress has been made in the development of chemical methods for the determination of choline and acetylcholine. The radioenzymatic methods (Sæters, Winkler and Simke 1970, Reid, Haubrich and Krishna 1971, Goldberg and McCaman 1973) and the gaschromatographic-massspectrometric methods (Jenden 1973, Karlén *et al.* 1974) should be mentioned. It was, however, felt that the measurement of acetylcholine released in intestine would be greatly aided by simpler or more inexpensive methods.

An approach in which nerve-containing preparations are incubated with radioactively labelled choline in order to mark the transmitter stores of the nerves with labelled acetylcholine has been used in metabolic studies of the rat diaphragm (Sæters and Stoll 1964, *et al.* 1970), the cat heart (Wallach, Goldberg and Shideman 1967), the cat superior cervical ganglion (Friesen, Kemp and Woodbury 1965, Collier and Lang 1969, Collier and MacIntosh 1970) and the rat cerebral cortex (Browning and Schulman 1968, Richardson and Szerb 1970). More recently this technique has also been applied in the guinea pig ileum (Szerb *et al.* 1970, 1976).

In the present study was undertaken with the intention of investigating if the use of radioactive tracers could substitute for other methods in the measurement of acetylcholine released from guinea pig ileum. The preparation was incubated with ^3H -choline of high specific activity whereafter the release of ^3H -acetylcholine could be studied.

Methods

Guinea pigs weighing 400–500 g were killed by blow on the neck and exsanguinated. A 10 cm long segment of the intestine was removed, beginning 10 cm proximal to the ileocecal sphincter and freed from surrounding fat by dissection. The longitudinal muscle with its adherent nerve plexus was prepared as described previously (Wikberg 1976). Preparations weighing about 40–60 mg are mounted in a wire jacketed glass chamber with capacity of 4 ml, containing Krebs solution of the following composition (in mM): NaCl 122.6, KCl 4.7, MgCl₂ 1.2, CaCl₂ 1.5, NaHCO₃ 15.4, KH₂PO₄ 1.2, glucose 5.5 and oxygen 0.03. The buffer is equilibrated at 37°C by gas mixture of 95% O₂ and 5% CO₂. The preparation was given a total volume of 5×10^{-4} M, the tension being recorded isometrically with Grass FT 03 transducer connected to a Grass polygraph. The organ bath had two 20 mm platinum electrodes mounted in its wall, 10 mm apart, parallel to the unopposed preparation.

The preparation was incubated with 20 μCi ^3H -choline (methyl ^3H -choline chloride 10.1 Ci/mmol, Amersham Chemical Centre, Amersham, England) at final concentration of 1×10^{-4} M, for 1 h. It was then added briefly to the Krebs solution, hereafter 4 ml fractions were collected in 5 min periods for subsequent analysis. In some experiments the preparation was stimulated electrically by 30–1000 rectangular pulses of duration of 1 ms and frequency of 0.5–10 Hz, generated by Grass SD9 stimulator. The stimulation was checked with Tektronix 5103N oscilloscope and the pulses delivered caused current of 120 mA at 15 V. This stimulation has previously been found to be supramaximal with regard to the isometric twitch response of the longitudinal guinea pig ileum (Wikberg 1976). After completion of these procedures the preparation was decanted and dissolved in 0.5 ml Soluene 350 (Packard Instrument Company Inc.), and assayed in 15 ml of toluene-based scintillation cocktail.

biochemical errors

In fractions were immediately acidified by the addition of 25 μl of 2 N HCl to bring the pH to about 4–5 and assayed in an ion bath. From the samples aliquots of 0.5 ml were taken and converted to 10 ml toluene-

Release of ³H-Acetylcholine from Isolated Guinea Pig Ileum. A Radiochemical Method for Studying the Release of the Cholinergic Neurotransmitter in the Intestine

By

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Abstract

WIKBERG J. Release of ³H-acetylcholine from isolated guinea pig ileum. A radiochemical method for studying the release of the cholinergic neurotransmitter in the intestine. *Acta physiol. scand* 1977 101 302-317

Innervated strips of longitudinal muscle of guinea pig ileum were incubated with ³H-choline in the presence of physostigmine after which the efflux of tritiated metabolites was studied. Under resting conditions ³H-acetylcholine, ³H-choline and other tritiated metabolites were released into the incubation media. Analysis of the kinetics of the release of the metabolites indicated that the release occurred from at least 2 different compartments. Under the unstimulated conditions used, between 7% and 78% of the radioactivity remained in a third non-releasable compartment. The release of ³H-acetylcholine showed spontaneous fluctuations which were positively correlated to spontaneous variations of the isometric tension of the preparation. Electrical field stimulation at low frequency (0.5 Hz) consistently increased the release of ³H-acetylcholine. The release of ³H-choline was increased only slightly or not at all by the electrical stimulation. For studying stimulation-evoked release of ³H-acetylcholine it is suggested that the assessment of the total quaternary amine release in addition to measurement of ³H-acetylcholine release. This approach considerably simplified the method. During 3 consecutive stimulation periods of 1 min each, the increase in the release of total quaternary amines diminished with time. By taking into account the reduction of the stores of radioactivity in the tissue during the experiment, correction for the age-related trend could be made. When the preparation was stimulated with between 30 and 3 000 pulses a close correlation was found between the increase in the release of total quaternary amines and the number of pulses delivered. The volley output of the quaternary amines was not constant, however, but diminished with increasing stimulation frequency.

Key words: Acetylcholine release, ileum, isotope labelling.

In studies of cholinergic mechanisms, the isolated mammalian intestine has often been utilized among other experimental systems. When for different reasons, acetylcholine has been measured, a biological assay method has generally been chosen. Choline can also be determined after acetylation to acetylcholine (Hunt 1915). The biological methods are sufficiently sensitive for most applications but some problems are encountered. The precision and specificity of these methods can be questioned. The assays may be influenced by a large number of factors, such as the presence of drugs or active substances.

$$f(A, M) = \sum_{j=0}^{\infty} (e^{-q_j M} - 1) A - q_j M \quad (2)$$

It should be noted that the non-releasable store, R , does not appear in equation (2). The experimental data are fitted to these equations by non-linear least square regression. This is achieved through linearizations of the models by Taylor series expansions and solving for the constants by multiple linear regression in an iterative algorithm. Draper and Smith (1966) give the outline of the mathematical basis. For this purpose, general regression programmes as written in BASIC, which had the capacity of estimating 20 constants of an arbitrary function.

The release of radioactivity to the fractions is summed from $t = 0$ to ∞ , then

$$\lim_{M \rightarrow \infty} \sum_{j=0}^{\infty} \sum_{i=0}^{\infty} (e^{-q_j M} - 1) A - q_j M = \sum_{j=0}^{\infty} A \quad (3)$$

the sum of all A represents the total amount of radioactivity that can be released from the preparation. The analysis of the stimulation-evoked release of radioactivity by this method of presenting the data is found. The release of total quaternary amines was calculated as

$$\Delta TQA_0 = TQA_{\text{stim}} - TQA_{\text{control}}$$

the unstimulated release of total quaternary amines (TQA) as the release just prior to the electrical stimulation. ΔTQA_0 was expressed in per cent of the total amount of radioactivity calculated to be present in the tissue at the beginning of the exp. This value could be regarded as uncorrected with respect to the release of radioactivity that could be released at the moment of stimulation. Correction is made by dividing the stimulation-evoked release in per cent of the stores of radioactivity (M) calculated to be present in the tissue at the beginning of the stimulation period

$$\Delta TQA = \frac{\Delta TQA_0}{M} \cdot 100$$

M is expressed in per cent of the amount at the beginning of the exp. (M_0). An essentially similar method of correcting the data has previously been applied by others to analyses of H-norepinephrine in electrical stimulation of adrenergically innervated organs (Sjölund and Brandan 1976).

Results

Release of labelled metabolites released from the unstimulated guinea pig ileum

After incubation with H-choline, the preparation was washed for 30 min with 6 bath volumes of Krebs solution, and fractions of the solution were then collected for analysis. Radioactivity was released spontaneously from the preparation at that time. Direct application of one ml of the washing solution on the Amberlite column yielded 3 peaks. One peak occurred between 2.5 and 7.5 ml in the void volume (6.8 ml) of the column, and this peak eluted with water. It will be referred to hereafter as the unbound fraction (UBF). The second peak appeared after 9–14 ml and the third after 15–21 ml of 0.1 M phosphate buffer (1.8 (Fig. 1A). These peaks had the same retention volumes as native acetylcholine and choline, respectively. Under resting conditions $24.2 \pm 6.9\%$ of the activity appeared in the unbound fraction and $38.9 \pm 4.4\%$ and $36.9 \pm 5.8\%$ in the acetylcholine and choline peaks, respectively (S.E. of mean, $n = 12$). After precipitation of the tissue perfusate with saturated ammonium molybdate as described under Methods (p. 304), and batch treatment with 1 M-equivalent Boehr 9 in 1 ml water no activity could be eluted with water after the application of the sample on the Amberlite column. The acetylcholine and choline peaks were still present and were eluted by the phosphate buffer after this procedure.

or a Triton-based scintillation cocktail. A 2 ml sample was taken for paper chromatography but to the rest of the sample 0.5 μ mol acetylcholine chloride and 0.5 μ mol choline chloride were added as carriers. The quaternary ammonium compounds were precipitated by the addition of 0.5 ml of a 10% solution of ammonium reineckate. After 1 h at +4°C the sample was centrifuged and the supernatant discarded. The pellet was washed once with 2 ml of a diluted solution of cold ammonium reineckate by repeated ammonium reineckate/water 1:5 (v/v) and centrifuged. The pellet was then batch treated with 10 m-equiv of Biorex 9 chloride form (Bio Rad Laboratories), in 2 ml ethanol at room temperature and after centrifugation the ethanol was poured into scintillation vials. The ion exchanger was added once again with 1 ml ethanol which was added to the scintillation vial. 10 ml of the toluene-based scintillation fluid was added to the sample. The recovery of acetylcholine by this method was found to be $97.4 \pm 0.5\%$ of choline $93.9 \pm 0.4\%$ (S.E. of mean $n=10$ in both cases).

Paper chromatography To the sample that was to be submitted to paper chromatography 1 μ mol acetylcholine and 1 μ mol choline carrier were added. The sample was precipitated with ammonium reineckate as described above and washed once. The precipitate was then dissolved in 4 ml methanol and 0.1 m-equiv Biorex and transferred to new tubes. The methanol was evaporated in a stream of dried air at room temperature, dissolved in 15 μ l methanol and applied to Whatman No. 1 filter paper for ascending chromatography in a solvent system composed either of acetone/water/10 N HCl 100:20:1 (by vol) or butane-1-ol/ethanol/water/acetic acid 8:2:3:1 (by volume). The spots were localized by developing with iodine vapours, cut into pieces and put into scintillation vials. The radioactivity was eluted off the paper with 3 ml ethanol/water 1:1 (v/v) and counted after the addition of 12 ml Instagel.

Ion exchange chromatography This was used for separating some samples. Amberlite CG 50 II (400 mesh) was washed with 1 N HCl, with the double volume required to give a negative flame test for metal ions. It was then washed first with water until the pH exceeded 5.0 and then with 0.1 M sodium phosphate buffer pH 7.0, until the pH reached 7.0. Columns measuring 4.4–4.50 mm are packed with ion exchanger and equilibrated with the phosphate buffer until the pH of the effluent is equal to that of the influent. Before the sample was applied the column was washed with 5 ml of water. To one ml of the acidified tissue perfusate 0.5 μ mol choline chloride and 0.5 μ mol acetylcholine chloride carrier were added. The sample was applied on the column followed by 5 ml of water and was then eluted at 10 ml/min with the phosphate buffer. Fractions of 1.25 ml were collected and counted in 10 ml Instagel. The addition of the carriers was found to markedly increase the resolution of the chromatographic separation.

Liquid scintillation counting The samples were counted in a Packard Tri Carb 3375 liquid scintillation spectrometer in either of three scintillation systems. Water-containing samples were counted in Instagel (Packard Instrument Company Inc.) or a scintillation cocktail composed of 333 ml Triton X 100, 66 ml toluene, 5 g PPO and 0.1 g POPOP. Solubilized tissue samples and ethanol solutions were counted in toluene scintillator composed of 111 ml toluene, 5 g PPO and 0.3 g POPOP. The quenching was determined by the use of an internal standard.

5) *Synthesis of H-acetylcholine*

Labelled acetylcholine was synthesized from H-choline. Tritiated choline 20μ Ci, was added to acetic acid anhydride. The mixture was allowed to react for 3 h at room temperature, whereafter the acid anhydride was evaporated. The residue was dissolved in a small volume of methanol and applied to paper for chromatography in the acetone/water/10 N HCl system. The acetylcholine spot was located with a Berthold LB2723 thin layer chromatographic scanner. The spot was cut out and extracted from paper into methanol.

Theory and statistical methods

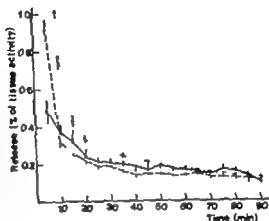
Let the amount of radioactivity in the tissue at time t be $A(t)$. If it is assumed that the disappearing radioactivity from the tissue follows a set of n exponential functions, but also that a non-releasable (B) of radioactivity is present in the tissue, we have

$$A(t) = \sum_i A_i e^{-\lambda_i t} + B$$

where A_i and λ_i are constants.

If the radioactivity released was collected in fractions during collection periods of equal duration the activity released during the j th collection period would be given by

2. Washout of radioactivity from the ileal plexus, pig ileum. The preparations were incubated with ^3H -cholesterol for 1 h. were then washed for 3-min periods and ileal metabolites are separated by means of perchloric acid and by paper chromatography. Release of acetylcholine (O O) and the unprecipitated fraction (—). The graphs show the S.E. of the mean from 3 different



the exp'ts. the release of radioactivity during a collection period was expressed in per cent of total amount of radioactivity calculated to be present in the tissue at the beginning of exp't. This is considered preferable to expressing the release in pmol/mg wet tissue for three reasons. Firstly the specific activity of the metabolites is not known since it might be diminished by dilution of endogenous substances. Thus it was not possible to relate the values with great accuracy by the latter method. Secondly the variation between most experiments was found to be smaller with the use of the percentual value, possibly to a normalization of the values by this method with respect to the actual amount of activity taken up by the tissue. Thirdly the percentual value has the advantage that it is directly to the amount of radioactivity that can be released from the preparation.

During the first washing period radioactivity was rapidly lost from the tissue. This was due to a rapid washout of ^3H -cholesterol, but also to a lesser extent to the release of the precipitated fraction. After the initial period the losses of all metabolites were less drastic.

Regression analysis. Analysis of the washout curves of ^3H -cholesterol and the unprecipitated fraction by the graphical curve peeling technique (Carter and Droogmans 1975) indicated that these curves consisted of two exponential decays. Analysis of the release of ^3H -acetylcholine by the same method was not as straightforward, this release showing greater fluctuations than that of the other metabolites. It could only be stated, therefore, that the release of ^3H -acetylcholine was probably best compatible with a biexponential model.

In the assumption that the release of all metabolites followed biexponential courses, the data were fitted into equation (2) by non-linear least square regression (see Methods, p. 305). The results of the determined constants are shown in Table I A.

The analysis indicated that the studied metabolites were released from 2 different compartments. (The two compartments of a particular metabolite were not, of course, necessarily the same as those of another metabolite.) It is not possible to arrive at a single physical interpretation of the organization of the compartments from the experimental data. Several different models would give rise to biexponential time courses in the release of radioactivity and no conclusions may be made, however, about the physical nature of the different compart-

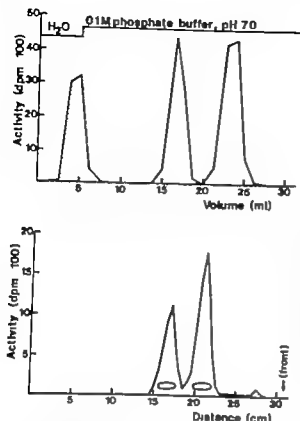


Fig. 1 A. Column chromatography of 1 ml of washing solution on Amberlite CO 50 B. The unbound fraction is eluted by water. The acetylcholine and choline peaks are eluted in 0.1 M phosphate buffer pH 7.0. B. Paper chromatography of total quaternary amine in acetone/water/10 N HCl (100:20:0). The chromatogram was cut into 0.5 cm wide strips and counted in Instagel after the radioactivity had been eluted off the paper by 3 ml ethanol (1 l). The peak of H-acetylcholine appears in front of the H-choline peak. The spots of free acetylcholine and choline are indicated below the peaks.

Separation of the ammonium-reineckate precipitated bath solution by paper chromatography yielded 2 peaks with acetone/water/10 N HCl with R_F values of 0.71 and 0.44. The R_F of native acetylcholine was found to be 0.69 and of choline 0.57 (Fig. 1 B). In butanol/acetone/water/ethanol/acetic acid, again two peaks appeared with R_F values of 0.46 and 0.36. The R_F of native acetylcholine was 0.46 and of choline 0.36. More than 95% of the radioactivity on the chromatograms was estimated to consist of choline and acetylcholine. Since the acetone/water/HCl system showed the most favourable R_F values, this system was used in all paper chromatographic separations in expts. reported below.

Kinetic of the release of tritiated metabolites under unstimulated conditions

Five minute fractions were collected for 1.5 h after the incubation period and the radioactivity in the Krebs solution was separated into 3 components. The TQA content was determined by the radioactivity in the reinecke precipitate of the wash solution. The free acetylcholine and H-choline in this fraction were separated by paper chromatography. The radioactivity of the metabolites which were left unprecipitated by ammonium reineckate was obtained by calculating the difference between the radioactivity of the washing solution and the TQA activity. In the following this fraction will be called the unprecipitated fraction (UPF).

After the incubation, 413 ± 46 pmol (788 ± 0.47 pmol/mg wet tissue) (S.E. of mean $n=5$) of the radioactivity was retained in the tissue. This amount corresponded to an uptake of 20.9 ± 2.3 % (S.E. of mean) of the total amount of radioactivity added to the incubation medium at the beginning of the expt.

II Estimates of extra- and intracellular compartments of radioactivity in the points p13 shown after incubation with ^3H -chole. The calculations are based on the data from Table I A under the assumption that the metabolites were released in extracell. systems.

	Extracellular ()	Intracellular ()
chole	5.74 ± 0.57	5.03 ± 0.27
chole	1.72 ± 0.38	10.8 ± 3.0
chole	1.07 ± 0.09	3.43 ± 0.93
unreleasable (by difference)	—	72.2 ± 5.6

at different times int. eq. (1) (see Methods, p. 304) on the assumption that the losses of metabolites could be approximated to a biexponential decay. The results are shown in Table I B, and Fig. 3. The non-releasable stores arrived at by the regression analysis were 1.13% (S.E. of mean), which was close to the value calculated above.

Correlation between H -acetylcholine release and isometric tension. Although exercise (10^{-4} M), which was present throughout the expt., induced a strong contraction of the preparation, spontaneous variations of the isometric tension were observed. Since the release of H -acetylcholine also showed spontaneous fluctuations, it was of interest to test the correlation between the two variables. As a suitable measure of the acetylcholine release, the residuals from the regressions were used. (A residual is the distance from a data point to the regression curve, see Draper and Smith 1966.) The deviation from the mean tension calculated for the whole experiment was used as measure of the isometric tension.

A strong positive correlation ($r = 0.64$, $p < 0.001$) between the two variables, from all the data points ($n = 90$) in five different expts., was found (Fig. 4).

Release of metabolites during electrical field stimulation

Studies of the kinetics of ^3H -chole release from the preincubated preparation indicated that essentially all extracellular metabolites had disappeared after a washing period of 30 min. For studying the effect of electrical stimulation, the preparation was therefore washed this length of time before any fractions were collected. After this 30 min 380 ± 37 pmol (21 ± 0.98 pmol/mg wet tissue) (S.E. of mean, $n = 5$) of the tracer was retained in the tissue. This amount corresponded to an uptake of $19.2 \pm 1.8\%$ (S.E. of mean) of the radioactivity present at the start of the incubation.

Separation of metabolites by paper chromatography. Electrical field stimulation by 150 pulses with a duration of 1 ms at 0.5 Hz given for 5 successive 5 min periods induced a large and consistent increase in the release of ^3H -acetylcholine ($75.0 \pm 15\%$ (S.E. of mean, $n = 25$) of the total increase of radioactivity induced by the electrical stimulation) (Fig. 5). The electrical stimulation also resulted in some increase in UPF amounting to $18.0 \pm 1.2\%$ (S.E. of mean, $n = 25$) of the total increase. Only a minor increase in the release of ^3H -chole ($7.0 \pm 1.3\%$ (S.E. of mean, $n = 25$) of the total increase) occurred on electrical stimulation.

Separation of metabolites by ion exchange chromatography. Separation of the metabolites by ion exchange chromatography gave similar results as paper chromatography. Electrical

TABLE I A. Estimated constants of equation (2) derived from nonlinear least square regression of expts. of choline, acetylcholine and UPF. B. Estimates of constants of equation (1) by activity calculated to be present in the tissue during the washout expt. (The expt. was formed under unstimulated conditions.)

Metabolite	A (%)	α (min ⁻¹)	A ₂ (%)	α (min ⁻¹)	31.
A					
Choline	3.30 ± 0.35	0.378 ± 0.009	5.47 ± 0.23	0.0150 ± 0.001	—
Acetylcholine	0.374 ± 0.067	0.120 ± 0.018	11.9 ± 4.9	6.40 ± 1.18 · 10 ⁻³	—
UPF	0.805 ± 0.078	0.388 ± 0.030	5.70 ± 0.93	9.54 ± 1.92 · 10 ⁻³	—
B					
Total	4.76 ± 0.37	0.303 ± 0.009	17.1 ± 0.98	0.0102 ± 0.0010	31.

ments. It is probable that the initial rapid release was due to a washout of the extracellular space. The slower components might be derived from intracellular compartments. Under certain assumptions concerning the models, it might be possible to arrive at a single set for the magnitude of the different compartments and the velocity constants of exchange of metabolites between the compartments and between the compartments and the surrounding solution. Owing to the uncertainty of what assumptions would be most compatible with reality however it is not possible to argue much on this point. The amount of radioactivity present in the compartments at $t=0$ will, however, be discussed. If one compartment is located extracellularly while the other was confined to the interior of the cells, the release of the metabolite from the intracellular compartment would probably have occurred into the extracellular space. The extracellular space would then release its activity to the surrounding Krebs solution. In this case the release would occur in a series, and a catenary system would then be present. The solution of a two-compartment catenary system was quoted by Othman and Droogmans (1975) from Huxley (1960). The amount of radioactivity present in the different compartments, as calculated by these formulas, is shown in Table II. In all cases the radioactivity present in the intracellular compartments was greater than that in the extracellular one. The largest pool was found in the intracellular acetylcholine depot. Of the extracellular radioactivity the largest amount was found in the choline fraction. No qualitatively different results from these would be derived if it was assumed that a parallel (mammary) system was present instead of a catenary one. In the mammary system the amount of radioactivity in a compartment at $t=0$ would be given by the A_i values in Table I A.

Without further arguing for or against any particular physical model of the observed relations it is possible to derive more information concerning the compartmentalization of radioactivity in the tissue. Irrespective of which model is chosen, the sum of all A_i values from the regressions can be shown to represent the total amount of radioactivity present in all releasable compartments at $t=0$ (see Methods, p. 305 eq. 3). This amount was calculated to be 27.7 ± 5.6% (S.E. of mean). Thus 72.2% of the initial 100% of radioactivity in the tissue was lacking, which meant that a rather large amount of radioactivity was present in unstimulated condition prevailing, consisted of non-releasable stores. This was further supported by fitting the data from the (calculated) amount of total radioactivity present

II Estimates of extra- and intracellular compartments of radioactivity in the guinea pig ileum after incubation with ^3H -choline. The calculations were based on the data from Table I A under the assumption that the metabolites are released in catalytic systems.

Extracellular (n) Intracellular (n)

choline	3.74 ± 0.37	5.03 ± 0.22
	1.72 ± 0.38	10.8 ± 5.0
	1.07 ± 0.09	5.43 ± 0.93
releasable (by difference)	—	72.2 ± 5.6

at different times into eq. (1) (see Methods, p. 304) on the assumption that the losses of metabolites could be approximated to a biexponential decay. The results are shown in Table I B, and Fig. 3. The non-releasable stores arrived at by the regression analysis were 13% (S.E. of mean), which was close to the value calculated above.

Relationship between *N*-acetylcholine release and isometric tension. Although eserine (10^{-4} M), which was present throughout the expt., induced a strong contraction of the intestine, spontaneous variations of the isometric tension were observed. Since the release of *N*-acetylcholine also showed spontaneous fluctuations, it was of interest to test the correlation between the two variables. As a suitable measure of the acetylcholine release, the residuals from the regressions were used. (A residual is the distance from a data point to the regression curve, see Draper and Smith 1966.) The deviation from the mean tension calculated for the whole experiment was used as measure of the isometric tension.

A strong positive correlation ($r = 0.64$, $P < 0.001$) between the two variables, from all the data points ($n = 90$) in five different expts., was found (Fig. 4).

Release of metabolites during electrical field stimulation

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For studying the effect of electrical stimulation, the preparation was therefore washed for a length of time before any fractions were collected. After this 30 min 380 ± 37 pmol (0.96 pmol/mg wet tissue) (S.E. of mean, $n = 5$) of the tracer was retained in the tissue. This amount corresponded to an uptake of $19.2 \pm 1.8\%$ (S.E. of mean) of the radioactivity present at the start of the incubation.

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Metabolite	A_1 (%)	α_1 (min ⁻¹)	A_2 (%)	α_2 (min ⁻¹)	ΣA_i
A					
Choline	3.30 ± 0.35	0.378 ± 0.009	5.47 ± 0.23	0.0150 ± 0.001	—
Acetylcholine	0.574 ± 0.067	0.120 ± 0.018	11.9 ± 4.9	$6.40 \pm 2.18 \cdot 10^{-3}$	—
UPF	0.805 ± 0.078	0.388 ± 0.030	5.70 ± 0.93	$9.54 \pm 1.9 \cdot 10^{-3}$	—
B					
Total	4.76 ± 0.37	0.303 ± 0.009	17.1 ± 0.98	0.0102 ± 0.0010	21.1

ments. It is probable that the initial rapid release was due to a washout of the extracellular space. The slower components might be derived from intracellular compartments. Under certain assumptions concerning the models, it might be possible to arrive at a single value for the magnitude of the different compartments and the velocity constants of exchange of metabolites between the compartments and between the compartments and the extracellular solution. Owing to the uncertainty of what assumptions would be most compatible with reality however it is not possible to argue much on this point. The amount of radioactivity present in the compartments at $t=0$ will, however, be discussed. If one compartment is located extracellularly while the other was confined to the interior of the cells, the release of the metabolite from the intracellular compartment would probably have occurred into the extracellular space. The extracellular space would then release its activity to the surrounding Krebs solution. In this case the release would occur in a series, and a catenary system would then be present. The solution of a two-compartment catenary system was quoted by Canel and Droogmans (1975) from Huxley (1960). The amount of radioactivity present in the different compartments, as calculated by these formulas, is shown in Table II. In all cases the radioactivity present in the intracellular compartments was greater than that in the extracellular one. The largest pool was found in the intracellular acetylcholine depot. Of the extracellular radioactivity the largest amount was found in the choline fraction. No qualitatively different results from these would be derived if it was assumed that a parallel (mammary) system was present instead of a catenary one. In the mammary system the amount present in a compartment at $t=0$ would be given by the A_i values in Table I A.

Without further arguing for or against any particular physical model of the observed relations it is possible to derive more information concerning the compartmentalization of radioactivity in the tissue. Irrespective of which model is chosen, the sum of all A_i values from the regressions can be shown to represent the total amount of radioactivity present in all releasable compartments at $t=0$ (see Methods, p. 305 eq. 3). This amount was calculated to be $27.7 \pm 5.6\%$ (S.E. of mean). Thus 72.2% of the initial 100% of radioactivity in the tissue was lacking, which meant that a rather large amount of radioactivity under unstimulated condition prevailing, consisted of non-releasable stores. This was further supported by fitting the data from the (calculated) amount of total radioactivity present in

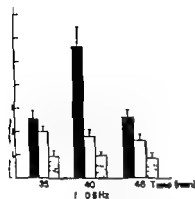


Fig. 6

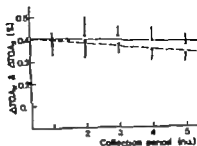


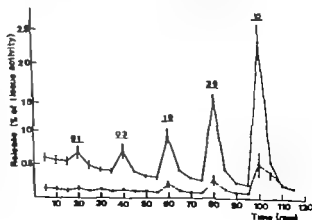
Fig. 7

6. Washout of radioactivity from the preincubated guinea pig ileum. The metabolites were separated by thin layer chromatography on Amberlite CG 30 II. Acetylcholine is indicated by filled, choline by unfilled and the unlabeled fraction by shaded columns. Electrical field stimulation at 5 Hz is performed during each collection period (—5).

7. Release of acetylcholine due to stimulation calculated as ΔTQA_0 (—) and calculated as ΔTQA_e (---).

Fig. 6 shows the washout of radioactivity from the preparation described above. ΔTQA calculated as a percent of the total radioactivity present in the tissue at the beginning of the expt. showed a slight decrease during the course of the stimulation periods. This is of course to be expected, but if, for instance, the effects of drugs were to be studied, it would be desirable that ΔTQA remained constant. By instead, calculating the release as the ΔTQA in per cent of the radioactivity present in the tissue at the beginning of the stimulation period, the release remained constant (Fig. 7).

Correlation between number of pulses delivered and ΔTQA . To investigate whether there was any correlation between the number of pulses delivered to the preparation and the release of ΔTQA , the preparation was stimulated by 30, 90, 300, 900 and 3 000 pulses with a duration of 1 ms for 5 min. Even the smallest number of pulses induced a clear increase of the ΔTQA .



8. Washout of radioactivity from the preincubated preparation. The metabolites are separated by precipitation with radioactivity. ΔTQA (—) and the unlabeled fraction (---). Electrical field stimulation at 5, 10, 15 and 30 Hz for 5 min as indicated by horizontal bars.

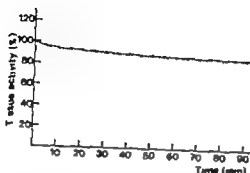


Fig. 3

Fig. 3 Loss of radioactivity from guinea pig ileum preincubated with H -choline. The preparation was washed for 5-min periods and the radioactivity present in the tissue was calculated from the counts released to the washing solution. The continuous line represents the regression of equation (1) in table 1.

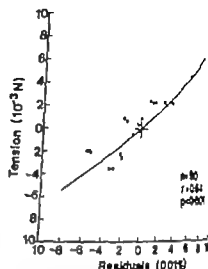


Fig. 4

Fig. 4 Correlation between spontaneous release of H -acetylcholine and the isometric tension of its preparation. The acetylcholine release was expressed as the deviation from the regression of the control curves (e.g. as the residuals from the regressions). The tension was expressed as the deviation from the mean tension in each expt.

field stimulation by 150 pulses with a duration of 1 ms at 0.5 Hz induced an increase in the release of acetylcholine similar to that in the previous expt. The electrical field stimulation did not affect the release of H -choline while the unbound fraction showed a slight tendency of increase (Fig. 6).

Measurement of stimulation evoked acetylcholine release as ΔTQA The expts. described above indicated that measurement of TQA was of equal value to measurement of acetylcholine itself in estimating the stimulation evoked release of acetylcholine, the reason being that the release of choline was almost unaffected by the electrical stimulation. In Fig. 7 the magnitude of the increase in TQA (ΔTQA) due to stimulation by 150 pulses at 0.5 Hz is

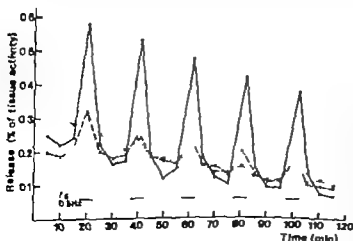


Fig. 5 Washout of radioactivity from the preincubated guinea pig ileum. The metabolites were separated by precipitation with reineckate and by paper chromatography. Acetylcholine (O—O) and the precipitated fraction (—) The total field stimulation at 0.5 Hz for 5 min (—) ($h=5$).

acetylcholine

reaction induced by low-frequency field stimulation in the longitudinal guinea pig ileal is known to be mediated by stimulation of cholinergic neurons. It has been observed these contractions diminish in amplitude after prolonged stimulation periods. If choline (1 mM) is added to the incubation medium, the contractions can be restored to their initial levels (Kosterlitz, Lydon and Watt 1970). These results might indicate that extracellular choline is an important precursor for the synthesis of acetylcholine by the nerves and the metabolism of the preparation itself is not sufficient to supply the nerves with acetylcholine.

The present study demonstrates that the guinea pig ileum takes up tritium when it is incubated with ^3H -choline. If the isotope is distributed equally in the tissue and the incubation medium, the expected uptake may be estimated to be roughly 1%. The value obtained experimentally however was approximately 20%, indicating that the uptake is an active process.

From absolute figures, about 7.9 pmol of radioactivity per mg wet weight was taken up and released by the tissue after 1 h of incubation with $1\ \mu\text{M}$ ^3H -choline. Szerb (1975) arrived at a similar result. He calculated that 8.8 pmol radioactivity per mg was taken up by the longitudinal guinea pig ileum after perfusion for 54 min with ^3H -choline at the same concentration as was used in the present experiments. The slightly lower uptake in the present study might be due to the fact that the preparation was incubated with a smaller amount of ^3H -choline than in the experiment of Szerb. From his results, the contents of endogenous acetylcholine and choline of the preparation could be calculated to be about 100 and 29 pmol/mg, respectively. No acetylcholine was present. A similar acetylcholine content (120 pmol/mg), could be calculated from the results of Paton and Zar (1968). The difference between the amount of endogenous metabolites and the amount of radioactivity retained in the tissue indicates that the label was probably diluted to a large extent by endogenous substances. This also indicates that there is little justification for expressing the release of radioactive metabolites as absolute figures as long as their specific activities are not known. The problem could perhaps be overcome by incubating the preparation at higher concentrations of ^3H -choline, thereby supposing that the dilution would become negligible. However since the sole purpose of the present study was to find a method for monitoring changes of acetylcholine release, no such attempts were made.

release of ^3H -acetylcholine

The present study confirms the results of Szerb (1976) that the preparation preincubated with ^3H -choline released ^3H -acetylcholine both during rest and on electrical stimulation when acetylcholine was present. In this work the identity of the ^3H -acetylcholine was established with the doublet, since the metabolite showed identical behaviour to that of native acetylcholine in three chromatographic systems. Thus, it was demonstrated that choline could serve as a precursor in the synthesis of acetylcholine in the guinea pig ileum.

Evidence has been presented that two choline transport systems are present in the guinea pig ileum. On incubation with ^3H -choline a high affinity site with a K_m of 2-4 μM and a low affinity site with K_m of 90-100 μM was observed. Furthermore, the high affinity

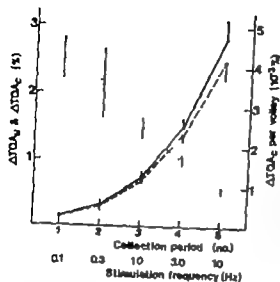


Fig. 9 Release of acetylcholine due to electric stimulation, calculated as ΔTQA (—), $\Delta TQA/v$ (---) and as ΔTQA released per volley (○) during stimulation at 0.1, 0.3, 1, 3 and 10 Hz for 3 min in 5.

release (Fig. 8). On increasing the number of shocks delivered, a close correlation to increase in the release of TQA was observed. The data were also corrected for loss of tritium by the method described above. The results are shown in Fig. 9 together with uncorrected data for comparison.

It has been shown by Paton and Zar (1968) that by increasing the stimulation frequency in the guinea pig ileum an increase in the total release of acetylcholine is obtained. If the release of acetylcholine is calculated, instead, as the output per delivered pulse, the result is in contrast smaller at high stimulation rates than at low. A similar observation was made in the present experiment, i.e. the release of ΔTQA per volley diminished at higher stimulation rates (Fig. 9).

Discussion

This investigation was started with the intention of finding a simple and fast method for studying acetylcholine release from the mammalian intestine. For reasons pointed out in the introduction, bioassay was excluded. As H -choline of high specific activity could be obtained, another approach became possible. It seemed that if the acetylcholine stores of the nerves in the preparation could be labelled by H -acetylcholine, detection of the neurotransmitter release might be fairly simple.

Isolated longitudinal guinea pig ileum was used for the study. This organ was found to be well suited for investigating cholinergic mechanisms by the radiochemical method. The preparation consists of the main parts of the Auerbach's plexus adherent to the longitudinal smooth muscle. A few circular muscle fibres may also sometimes be observed (Paton and Zar 1968; Wikberg 1976). Thus, the only part of the intestinal nerve plexus that is studied in this preparation is the nervous network between the longitudinal and circular muscle. Other tissue layers such as the intestinal mucosa are not present; otherwise these would probably have introduced difficulties in interpretation of the results. The presence of smooth muscle in addition to the nerves, must however be taken into account.

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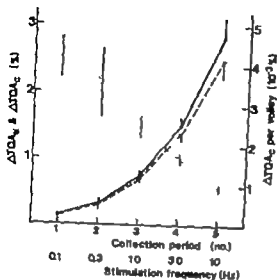


Fig. 9 Release of acetylcholine due to direct stimulation, calculated as ΔTQA_0 (—) $\Delta TQA_0 / (\Delta TQA_0 + \Delta TQA)$ and as ΔTQA_0 released per volley (---) for stimulation at 0.1, 0.3, 1, 3, and 10 Hz for 5 min.

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as strong as for the other metabolites. It is probable that the metabolites were released during the incubation, thus accumulating extracellularly. This accumulation of extracellular metabolites might then be the basis of the rapid phase of the efflux. The amounts of radioactivity in the acetylcholine and in the unprecipitated fraction, present in the extracellular medium, seemed to be small in comparison with the other pools of radioactivity. It is suggested that the slow components of the release are derived from intracellular sites. The release of acetylcholine from this compartment is discussed further below. The mechanism of the release of the metabolites in the unprecipitated fraction is unknown. The release is perhaps derived from metabolism of more tightly bound stores of radioactivity in tissue.

Between 72 and 78% of the radioactivity in the unstimulated preparation, consisted of releasable stores. This finding should not necessarily mean that the stores were strictly releasable; thus, this compartment might represent a release of radioactivity with a very long half time. During a short period (1.5 h) such a pool would appear as non-releasable. However, these stores were observed as a tightly bound pool of radioactivity in the tissue. Whether or not this pool can be released upon prolonged electrical stimulation remains to be established. Szerb (1976) suggested that two populations of cholinergic neurons were present in guinea pig ileum. One of these populations released its acetylcholine content under spontaneous conditions or during low frequency stimulation. The other population is only activated at high rates of stimulation. The latter population of neurons might at least partly be responsible for the non-releasable store observed in the present work.

It is also possible that the non-releasable stores consisted of choline incorporated into phospholipids, suggested by Collier and Lang (1969) to be synthesized from H-choline in sympathetic ganglions. However the trichloroacetic acid insoluble fraction which appeared after 54 min of incubation with ^3H -choline at $1 \mu\text{M}$ in the study of Szerb (1975), seems to be too small to completely account for the non-releasable pool in the present experiment.

Spontaneous release

It is well known that intestinal preparations have spontaneous contractile activity of varying intensity and frequency. In the guinea pig ileum these contractions, when they occur are stimulated by eserine ($3 \cdot 10^{-6} \text{ M}$) and blocked by tropine ($3.5 \cdot 10^{-6} \text{ M}$) (Wolberg, unpublished observations), which indicated that the parasympathetic system plays a role in the spontaneous intestinal motility of this organ. Under unstimulated conditions the release of acetylcholine showed spontaneous fluctuations. The variations were much greater than the variation in the release of ^3H -choline or of the unprecipitated fraction. It is not improbable that the spontaneous fluctuations of the acetylcholine release could be due to spontaneous variations in the activity of the cholinergic neurons of the preparation. A good correlation between the release of acetylcholine and the mechanical tension was also observed. It is found possible to arrive at this correlation even though the strong contraction induced by eserine probably obscured some of the variations of the intestinal tone. These observations combined make a fairly coherent picture and indicate the consistency of the radiochemical method for the detection of ^3H -acetylcholine.

site disappeared when the preparation was denervated while the low affinity site was present after this procedure (Pert and Snyder 1974). In a study of the present type it was desirable for the acetylcholine stores of the nerves to be selectively labelled, as this would reduce the disturbance from other metabolites released from the preparation. The data of Pert and Snyder indicate that a low concentration (0.4–1 μM) of ^3H -choline should be used when incubating the preparation, in order to take advantage of the high affinity transport system of the neurons. Both their results and the results of Szerb (1975) indicate that this will lead to a larger synthesis of ^3H -acetylcholine relative to other metabolites, than when high concentrations (50–100 μM) of ^3H -choline are used.

Other metabolites Besides choline and acetylcholine other metabolites were also released from the preparation. It is probable that these metabolites, which were named bound and unbound, represented the same substances, since the bound fraction disappeared after precipitation by reineckate.

At the present moment the identity of the unbound and the unprecipitated fractions is not known. Their nature may be speculated upon, however. They are probably not simply positively charged, since they were not bound by the Amberlite column. It is also probable that they are not quaternary cations, since these might be expected to be precipitated by reineckate ions. Possible candidates for the metabolites are therefore less strongly charged molecules such as N, N dimethylethanol-amine or zwitterions such as phosphorylcholine and betaine. Szerb (1976) suggested that the fraction remaining after precipitation with reineckate, which was released from guinea pig ileum, represented ^3H -phosphorylcholine, since this metabolite was identified in extracts from preparations preincubated with ^3H -choline. The definite settlement of the nature of UPF should, however, await its positive chemical identification in perfusate.

Compartmentalization of tritiated metabolites From the kinetic analysis of the release of ^3H -choline it was apparent that the metabolite was released with a biexponential time course, the half times being 1.8 and 46 min respectively. It is suggested that the metabolite was released from two different compartments, an extracellular and an intracellular. It is not understandable why ^3H -choline should be released in large quantities from the extracellular space. This extracellular choline could represent the choline of the incubation medium which was not taken up by the tissue and not washed away by the first brief rinse of the preparation. The source of the slow component of choline release is at present not clear. This could be derived from the nerves as well as from the smooth muscle of the preparation. It could represent a breakdown of acetylcholine by esterase not blocked by eserine. This choline could also be derived from an intracellular pool of free choline. It was suggested that such choline might have been present in the cat superior cervical ganglion after perfusion with tritiated choline (Collier and MacIntosh 1969).

Efflux studies on the isolated rat diaphragm indicated similar conditions to those in the guinea pig ileum. The rat diaphragm released ^3H -choline from two different compartments with half times of 2.5 and 36 min, when the preparation had been preincubated with labelled choline (Potter 1970).

There was little doubt that the unprecipitated fraction was released from two different compartments. This could also be true for acetylcholine, although the evidence for this

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In this work the spontaneous release of ^3H -acetylcholine ranged between 0.097–0.039% of the total stores of radioactivity present at the beginning of the experiment. From the data of Paton and Zar (1968) the release of acetylcholine in the longitudinal ileum can be calculated to be 0.34%/min of the acetylcholine content in the preparation. The acetylcholine release in the present study and that in the study by Paton and Zar are not strictly comparable, as they were calculated by different methods. However, the results do not seem to be grossly discrepant, since the release calculated in this work may be expected to be lower than the value derived from Paton and Zar (1968). The previous value refers not only to the acetylcholine content of the tissue but also to other metabolites present in the tissue.

Stimulation-evoked release Electrical field stimulation increased the release of ^3H -acetylcholine from the preparation. The ^3H -choline release was increased only slightly or not at all by the electrical stimulation and the release of the unknown metabolites may increase to some extent.

Since the electrical stimulation almost exclusively affected the release of the acetylcholine and not the release of the choline, and since the TQA was found to contain only three metabolites, it is suggested that measurement of TQA is of equal value to measurement of ^3H -acetylcholine in the study of stimulation-evoked release of the neurotransmitter. Measurement of TQA is preferable as the chromatographic procedures are rather time consuming. This approach will therefore considerably reduce the work involved in the method. However, it should be emphasized that under unstimulated conditions, determination of TQA is probably of limited value, as the partition of acetylcholine and choline cannot be predicted.

One of the most important findings in the present work was that the stores of radioactivity in the preparation, were sufficiently great to allow repeated stimulation of the preparation and still obtain a large release of ^3H -acetylcholine. The release of ΔTQA decreased naturally slightly on repeated stimulation at constant frequency. By calculating the release of ^3H -acetylcholine as ΔTQA a correction was obtained, so that the release remained almost constant. This finding could be of importance in studies of the influence of drugs, for example, on the stimulation-evoked release of acetylcholine.

The release of total quaternary amines on electrical stimulation at different rates is similar to the release of acetylcholine under similar conditions reported by Paton and Zar (1968). The increase of ΔTQA was greater at high rates of stimulation, but the increase in volley diminished. The similarity between the results of this study and those of Paton and Zar strengthens the validity of the present method.

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- 2.3 capacity of mouse skeletal muscle to oxidize radioactively labelled fatty acid and the linkage of the fatty acid oxidation capacity to the other metabolic pathways. The study was first conducted with "mixed" skeletal muscle and then extended to comprise the effects of training in predominantly red and predominantly white muscle tissue.

Material and Methods

and training

Study (EXP I) was first conducted with young (2 months old at the beginning of training) male mice. The training animals (N=11) were running 30 min/day 4 days a week during 5 months on a treadmill at a speed of 20 m/min. Control animals (N=9) lived in normal cage conditions. Second exp. (EXP II) male F₁-hybrids (B6D2F1/BOM, C1 Bombholtgård Ltd, Denmark) of the same age at the beginning of training were used. The training animals (N=35) were running 1 h 7 days a week, 3 times per day 30 min at a speed of 20.5 m/min, 45 min at a speed of 17.6 m/min and 15 min at a speed of 16.6 m/min. Control animals (N=27) lived in normal cage conditions. Third exp. (EXP III) male NMRI mice of the age of 2 months were used. The training group ran 5 days a week 60 min per day for 4 months at a speed of 17.6 m/min. Control animals lived in normal cage conditions.

Mice were given commercial pellet food (Hankkija, Finland) and water *ad libitum*. Animals were housed in 46 × 13 cm cages (8–10 mice in each cage) in a room where temperature and humidity constant. The day was artificially divided into 12 h of light and 12 h of darkness changing at 6:00 a.m. Training of animals was performed before noon on fixed time in each experimental series.

samples

Mice were trained up until the day before sacrifice. During each exp. day 2 or 3 control and trained mice were studied. The animals were sacrificed by dislocation of the neck and the muscle sample under study quickly excised and weighed. In EXP I and II the whole *musculus quadriceps femoris* (MQF) was used. In EXP III the right MQF was divided into grossly red and grossly white parts while the left MQF served as in EXP I and II. The grossly red part consisted of the proximal heads of *m. rectus femoris* and *m. vastus medialis* and the red fibers of *m. vastus lateralis*. The grossly red sample is not fully red because the superficial parts of *m. vastus medialis* and *m. vastus lateralis* contain also fibers. The grossly red part of control mice weighed 77.6 ± 3.7 (S.D.) mg and that of trained mice 119 mg. The predominantly white part consisted of the distal heads of *m. rectus femoris* and *m. vastus medialis*. In controls this sample weighed 66.9 ± 6.5 mg and in trained animals 66.2 ± 4.1 mg. The sample was cut with scissors into smaller pieces and homogenized in Porter Elvehjem homogenizer before putting in ice-cold buffer (175 mM KCl, 0.1 mM EDTA, pH 7.4). The homogenization was made with 4–6 complete passes of the pestle. The preparation was made 2 and an aliquot (1–30 ml) was removed for the measurement of fatty acid oxidation capacity (FAO).

method of fatty acid oxidation capacity

A glycol modified reaction mixture for FAO measurement (Mole *et al.* 1970) contained 5 mM MgCl₂, 10 mM KCl, 40 mM potassium phosphate buffer (pH 7.4), 2 mM EDTA, 25 μ M coenzyme A, 1 mM α -NADP, 2 mM ADP and 70 μ M palmitate as NH₄ salt. The proportion of carbon-14-labelled (New England Nuclear Corp.) palmitate was 5:1. The radioactivity of the substrate was in EXP I 300 000 dpm in EXP II and III 700 000 dpm per 0.11 μ mol palmitate.

The reaction was carried out in liquid scintillation counter bottles (250 ml), which were in water at 37°C under light shaking. The reaction was started by addition of 1.0 ml homogenate (20 mg wet wt) and the vessel was closed with rubber stopper. After 30 min the reaction was stopped by adding 1.0 ml 10% citric acid into the reaction bottle. The delivered carbon dioxide was washed by oxygen gas "trap" bottle containing 0.5 ml ethanethiolane-methylcellulose (1:1) absorbed on a piece of paper. At the beginning of 1.5 h measuring the oxygen flow rate was 10–15 ml/min and the flow was 7 accelerated during the shaking. The trap was very effective since over 95% of ¹⁴CO₂ was detected in the trap vessel already after 30 min flow. The filter paper was folded and 10 ml of scintillation liquid (10 ml PPO, 3 mg POPOP, 2 ml methylcellulose, 8 ml toluene) was added and the radioactivity was

Effect of Endurance Training on the Capacity of Red and White Skeletal Muscle of Mouse to Oxidize Carboxyl ^{14}C -labelled Palmitate

By

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Abstract

SALMINEN A, V VIHKO and L PILSTRÖM. Effect of endurance training on the capacity of red and white skeletal muscle of mouse to oxidize carboxyl- ^{14}C -labelled palmitate. *Acta physiol. scand* 1977 101 318-328.

Three groups of mice were trained for 1, 4 and 5 months according to different running protocols on a motor driven treadmill and the fatty acid oxidation capacity (FAO) and the activities of some energy metabolism (cytochrome c oxidase, malate dehydrogenase, triosephosphate dehydrogenase, lactate dehydrogenase) were determined from *m. quadriceps femoris* (MQF). Endurance training increased the FAO [3-month training, 4 days/week, 30 min/day $+2\%$ ($p < 0.05$), 1-month training, 7 d 150 min/day 37% ($p < 0.001$); 4-month training, 3 days/week, 60 min/day 4% ($p < 0.05$)]. The activities of cytochrome c oxidase and malate dehydrogenase increased approx. 30% ($p < 0.001$) whereas triosephosphate dehydrogenase and lactate dehydrogenase activities were not prominently influenced by training. The predominantly red part of MQF of untrained animals oxidized palmitate four times faster than the predominantly white part. The activities of cytochrome c oxidase and malate dehydrogenase were 10-15 times higher showing pronounced FAO in the red part. Endurance training increased the activities of oxidative enzymes in the red and white parts and the whole muscle retained the differences between the muscle types after training. The absolute increase in the red muscle was, however, manyfold when compared in chemical units to the white muscle.

Key words: Red/white skeletal muscle, palmitate oxidation, endurance training, enzyme activities.

Endurance training adapts the metabolic capacities of exercised skeletal muscles. An important adaptation is the increase in the oxidative capacity of muscle. This is reflected as increased activities of the enzymes of citric acid cycle (e.g. Holloszy 1967), β -oxidation (Mole *et al* 1971, Baldwin *et al* 1972) and respiratory chain (e.g. Holloszy 1967, Baldwin *et al* 1972).

Fatty acid oxidation provides the major part of energy utilized during aerobic energy performances (Keul *et al* 1972). In the mammalian skeletal muscle red fibers are specialized for oxidative energy production and they are recruited during prolonged exercise. In the following study we investigated the effects of endurance type of training

lactate dehydrogenase activity (LDH) (E.C. 1.1.1.27) was used as a measure for the anaerobic capacity of muscle. The assay was performed according to Kornberg (1955) by following the oxidation of NADH (0.2 mM) at 340 mμ in the presence of sodium pyruvate (5 mM) in 50 mM phosphate buffer (pH 7.5) and 10 μl homogenate dilution (1:10) in a final volume of 0.925 ml at 22°C.

Malate dehydrogenase activity (MDH) (E.C. 1.1.1.37) was, like CytOx, used as a marker for oxidative capacity. The reaction mixture (Ochoa 1955) contained 25 mM Tris-HCl pH 7.4, 0.5 mM ascorbate, 0.5 mM NADH and 25 μl homogenate dilution (1:10) in a final volume of 0.925 ml at 22°C.

ATPase activity (TPDH) (E.C. 1.2.1.12) was used as a measure for the aerobic capacity of the muscle. The reaction mixture (Bass et al. 1969) contained 4 mM EDTA, 40 mM Tris-HCl pH 7.5, 2.5 mM MgSO₄, 1.1 mM ATP, 5.1 mM D-3-phosphoglycerate, 1.8 mM glutathione, 0.16 mM NADH, 10 μl phosphoglycerate kinase (20 μg, Boehringer Mannheim) and 25 μl homogenate dilution (1:10) in a final volume of 0.685 ml at 22°C.

Muscle activities are referred per muscle wet weight and per protein mass. Protein concentrations were estimated according to Lowry et al. (1951). In EXP II the homogenates were hydrolyzed before the assay in 1 N NaOH at 40°C for 1 h. Bovine serum albumin (Sigma) was used as protein standard.

Statistical methods

Statistical procedures are used to calculate means and standard deviations (SD). The significance of differences between means were calculated by Student's *t*-test. Correlation coefficients between different data were determined by the least square method.

Results

Effects of endurance training on FAO in MQF

Endurance training increased the capacity of MQF of mouse to oxidize palmitate *in vitro*. In the pilot study (EXP I) the FAO of trained mice, expressed on protein basis, was higher ($p < 0.05$) than in controls. Per wet weight the difference was 23.5%, but not statistically significant (Table I).

The more intensive training of F₁-hybrid mice in EXP II caused markedly greater effects (Table II). The FAO of controls was 41.9 ± 6.6 and that of trained mice 57.2 ± 11.1 μmol/mg muscle wet weight. This difference was 36.5% ($p < 0.001$). When calculated on protein basis the increase was even higher (40%, $p < 0.001$). CytOx activity increased 46% ($p < 0.001$), i.e. less than FAO capacity. LDH activity decreased but the change was not small and significant only in case of activity per muscle wet weight (Table II).

In EXP III the FAO in MQF of controls was 41.7 ± 8.4 and that of trained 51.8 ± 14.9 μmol/mg wet weight. The increase was 24.2% ($p < 0.05$). CytOx ($p < 0.05$) and MDH

($p < 0.01$) EXP I. The weights of control and trained animals, the weights and protein contents of the quadriceps femoris (MQF) and the fatty acid oxidation rates (FAO) (mean \pm S.D.) together with the per cent differences between control and trained animals (*) and the significance (*p*) of the differences.

Table	Controls (n=9)	Trained (n=11)	%	
Weight of animals (g)	36.3 \pm 2.7	34.9 \pm 2.6	- 4.5	
Weight of MQF (mg)	271 \pm 23	260 \pm 24	- 4.2	
Protein content* of MQF	163 \pm 28	166 \pm 20	+ 1.8	
FAO/mg muscle wet weight	31.5 \pm 8.4	38.8 \pm 7.8	+ 23.5	*
FAO/mg muscle protein	192 \pm 43	235 \pm 43	+ 22.4	< .05

* Protein content: μg protein/mg muscle wet weight.

FAO: μmol/mg muscle wet weight or muscle protein.

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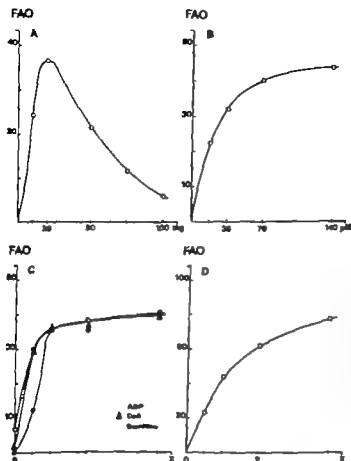


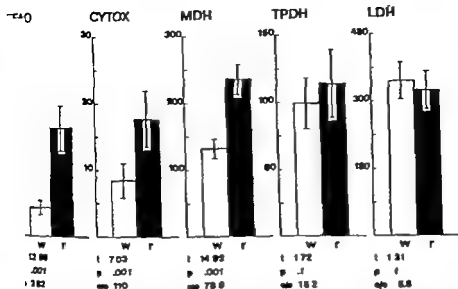
Fig. 1. Some testing results of FAO measurement. Values for FAO are expressed as μ mol/min/g muscle. The optimum amount of muscle is 20–30 mg (Fig. 1A). The effect of NH_4 -palmitate concentration on FAO is the routine assay (Fig. 1B). The effects of concentrations of cofactors (ADP, CoA, carnitine) on FAO. In the test series the concentration of one cofactor at a time has been doubled etc. while the others have been the same as in the routine assay (Fig. 1C, D). The effect of manifold concentrations of cofactors on FAO.

measured with a Wallac scintillation counter. Double assays per each muscle sample were assayed. The method error was approximately 5%.

In Fig. 1 some test results of the FAO measurement method are given. The highest FAO was achieved with 20 mg of muscle (Fig. 1A) when the protein content in the reaction mixture was approximately 2.5 mg/ml. Optimal reaction time was 30 min, longer incubation slightly decreased the FAO. A maximal oxidation rate was achieved by substrate concentration 70 μ M palmitate (Fig. 1B) which was used in the routine assays. Michaelis-Menten constant (K_m) was then approx. 15 μ M. The effects of ADP, CoA and carnitine on the oxidation rate were also checked (Fig. 1C, D). ADP was the most important activator. The cofactor concentrations used in the routine method gave about 50% of maximal oxidation rate (Fig. 1C), although manifold addition of ADP, CoA and carnitine slightly but also increased the oxidation rate (Fig. 1D). The homogenization procedure is very important for the FAO assay. Strong homogenization decreased the oxidation rate and low activities were also recorded if sample was minced with scissors only or tissue slices were used. Inhibition in oxidation rate was observed if homogenate was stored longer than 2 h at 0–4°C.

Assay of enzymatic activities

The homogenates of the whole MQF (but not predominantly red or white parts) were further homogenized for 1 min and used for enzyme assays. Cytochrome oxidase activity (Cytochrome oxidase, E.C. 1.9.3.1) for estimating the oxidative capacity of the muscle, was determined polarographically (Wharton, 1968) using a Clark-electrode. Into the reaction chamber 3.0 ml 50 mM phosphate buffer (pH 7.4, temperature 37°C) and 100 μ l homogenate were added. After few minutes 50 μ l of a mixture of 60 mM N,N,N',N' -tetramethylphenyldiamine and 120 mM sodium ascorbate was added and the blank was registered for few minutes. The reaction was started by the addition of 10 μ l 4 mM cytochrome c and registered for approx. 5 min.



The differences in the fatty acid oxidation capacity (FAO) and the activities of cytochrome c (CYTOX), malate dehydrogenase (MDH), triphosphatase dehydrogenase (TPDH) and lactate dehydrogenase (LDH) between the normally active (white columns) and severely red (black columns) parts of untrained NMRI mice in EXCP III. The figures below show the *t*-value for the difference, *n* for the significance of the difference and per cent difference in the activities between white and black tissue types.

EXCP III. The means and standard deviations of different variables in control and trained animals together with the per cent differences between groups and the significance of the difference. FAO and enzyme activities are referred per muscle wet weight.

Variable	Control (n=14)	Trained (n=13)	%	p
fat of animals	38.6 ± 1.7	34.6 ± 1.8	- 5.2	< .05
fat of MQP	256 ± 24	244 ± 23	- 4.7	n.s.
white part	17.2 ± 4.3	19.3 ± 6.6	+ 12.3	n.s.
red part	63.6 ± 13.8	80.8 ± 13.6	+ 23.2	< .01
MQP	41.7 ± 8.4	51.3 ± 14.9	+ 24.2	< .05
white part	8.4 ± 2.6	10.7 ± 3.2	+ 27.2	< .05
red part	17.7 ± 4.2	23.8 ± 6.6	+ 34.3	.01
MQP	18.0 ± 4.2	23.8 ± 7.1	+ 31.7	< .05
white part	132 ± 14	164 ± 31	+ 24.2	.01
red part	236 ± 22	289 ± 36	+ 22.3	< .001
MQP	171 ± 16	222 ± 28	+ 29.8	.001
white part	99 ± 19	111 ± 27	+ 12.1	n.s.
red part	115 ± 25	116 ± 17	+ 1.7	n.s.
MQP	103 ± 21	111 ± 23	+ 7.8	n.s.
white part	346 ± 41	357 ± 55	+ 3.2	n.s.
red part	326 ± 40	299 ± 53	- 8.3	
MQP	319 ± 39	314 ± 43	- 1.6	

Malate dehydrogenase and triphosphatase dehydrogenase activities are expressed as $\mu\text{mol/min/mg}$ wet weight at 22°C.

TABLE II EXP II The means and standard deviations of different variables in control and animals together with the per cent differences between the groups and the significance of the difference.

Variable	Control (n=27)	Trained (n=35)	%	p
Weight of animals	29.1 ± 1.9	29.7 ± 2.5	+ 1.9	NS
Weight of MQF	228 ± 19	236 ± 26	+ 3.7	NS
Protein content of MQF	193 ± 21	189 ± 24	- 2.0	NS
FAO/muscle wet weight	41.9 ± 6.6	57.2 ± 11.1	+ 36.5	<.001
FAO/muscle protein	220 ± 46	308 ± 81	+ 40.0	<.001
CytOx/muscle wet weight ^a	18.0 ± 4.9	23.6 ± 5.2	+ 30.6	<.001
CytOx/muscle protein ^a	94.0 ± 16.8	125.4 ± 28.4	+ 33.4	<.001
LDH/muscle wet weight ^b	246 ± 58	256 ± 51	+ 10.5	<.05
LDH/muscle protein ^b	1496 ± 345	1366 ± 300	- 8.7	NS

^a Cytochrome c oxidase activity as nmol O₂ consumed/min/mg at 28°C.

^b Lactate dehydrogenase activity as nmol NADH oxidized/min/mg at 22°C.

($p < 0.001$) activities increased slightly more (31.7 and 29.8%) than FAO. Endurance training had no significant effects on the anaerobic (LDH) or glycolytic (TPDH) capacities (Ta

Differences in FAO and enzyme activities between grossly red and grossly white muscle of untrained mice

The basic differences between white and red part of MQF of untrained mice in C₁ MDH, TPDH and LDH activities and in FAO are shown in Fig. 2. In the red part was 4 times higher than in the white part (65.6 ± 13.8 and 17.2 ± 4.3 dpm/min/g wet weight). CytOx and MDH activities in red tissue were twice as high as in the white ($p < 0.001$) whereas the differences in TPDH or LDH activities were not significant.

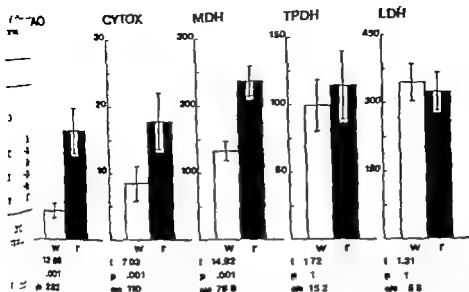
Effects of endurance training in grossly red and grossly white muscle tissue

The effects of endurance training on FAO and enzyme activities in red and white muscle and for comparison in the whole MQF (mixed) are given in Table III. The FAO in red tissue increased more (23.2%) than in white tissue (12.3%). In absolute terms the FAO in red muscle increased almost seven times more than in white tissue. CytOx and LDH activities increased more in red tissue although the per cent change was rather similar in both types. No clear effects of training were registered in the activities of TPDH or LDH.

The relationships between FAO and enzyme activities

The intercorrelations between FAO, CytOx and LDH in EXP II are given in Table IV. FAO correlated positively and highly significantly ($p < 0.001$) with CytOx. In untrained groups, separately, the correlation coefficients were lower. Correlations between anaerobic capacity (LDH) and aerobic (FAO or CytOx) were more random, most of them were negative.

The intercorrelations of FAO, CytOx, MDH, TPDH and LDH in EXP III are given in Table V for the both muscle types and for all the observations. The strongest correlations were observed between variables of oxidative metabolism (CytOx and MDH).



2. The differences in the fatty acid oxidation capacity (FAO) and the activities of cytochrome *c* (CYTOX), malate dehydrogenase (MDH), triosephosphate dehydrogenase (TPDH) and lactate dehydrogenase (LDH) between the grossly white (white columns) and grossly red (black columns) parts of the muscle were significant in EDP III. The figures below show the *t*-values for the difference, to test the significance of the difference and per cent difference in the activities between white and red muscle types.

EDP III The means and standard deviations of different variables in control and trained animals together with the per cent differences between groups and the significance of the difference. FAO and enzyme activities are referred per muscle wet weight.

	White	Control (n=14)	Trained (n=13)		
EDP I					
EDP II					
EDP III					
EDP IV					
EDP V					
EDP VI					
EDP VII					
EDP VIII					
EDP IX					
EDP X					
EDP XI					
EDP XII					
EDP XIII					
EDP XIV					
EDP XV					
EDP XVI					
EDP XVII					
EDP XVIII					
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EDP XXXI					
EDP XXXII					
EDP XXXIII					
EDP XXXIV					
EDP XXXV					
EDP XXXVI					
EDP XXXVII					
EDP XXXVIII					
EDP XXXIX					
EDP XL					
EDP XLI					
EDP XLII					
EDP XLIII					
EDP XLIV					
EDP XLV					
EDP XLVI					
EDP XLVII					
EDP XLVIII					
EDP XLIX					
EDP L					

FAO, fatty acid oxidation; MDH, malate dehydrogenase; TPDH, triosephosphate dehydrogenase; LDH, lactate dehydrogenase; CYTOX, cytochrome *c*. Activities are expressed as nmol/min/mg wet weight at 37°C.

TABLE IV Correlation coefficients between the measured variables in EXP II. The significance of correlation coefficients: $p < 0.05$, $p < 0.01$ and $p < 0.001$

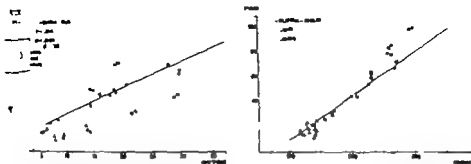
Group	CytOx/FAO	LDH/FAO	LDH/CytOx
Wet weight			
Controls	.026	.203	-.190
Trained	.231	-.244	-.327
Together	.464	-.237	-.164
Protein			
Controls	.354	.503	.050
Trained	.406	.163	-.130
Together	.576	.106	-.164

especially if all measured values from the different muscle samples are accounted (Fig. 3). Correlation coefficients were then statistically highly significant.

Another positive correlation pattern existed between TPDH and LDH activity ($p < 0.001$). In this case correlation coefficients were high also in separate muscle type (Table V). The relationships between TPDH and oxidative variables were weak or mostly positive. On the other hand, LDH activity correlated often negatively with oxidative variables. These correlations were statistically significant only in some cases.

TABLE V Correlation coefficients between the measured variables in EXP III in different muscle type separately in control and trained groups and the whole sample. Statistical significance as in Table IV

Muscle group	CytOx/FAO	MDH/FAO	TPDH/FAO	LDH/FAO	MDH/CytOx	TPDH/LDH/CytOx	TPDH/MDH	LDH/MDH	LDH/TPDH
White									
Controls	.342	.037	-.026	-.262	.306	.211	.164	.080	.011
Trained	.189	.325	.201	.062	.676	.240	.775	-.362	.409
Together	.293	.318	.178	-.024	.640*	.305	.543	-.072	.317
Red									
Controls	.077	.719	.165	-.097	-.153	.026	-.336	.428	.313
Trained	.098	-.142	.316	.371	.790*	.217	.430	.045	.359*
Together	.310	.474	.208	-.004	.672	.133	.008	.195	.146
Whole muscle									
Controls	.257	.164	-.055	-.062	.402	.179	.249	.094	.344
Trained	.137	.026	.270	.127	.857	.400	.611	.124	.386
Together	.326	.353	.203	.045	.777*	.362	.397	.210	.270
All values									
Controls	.643	.907	-.85	-.230	.580*	.230	-.214	.359	-.083
Trained	.613	.774	.177	-.306	.795	.224	.003	.035	-.122
Together	.638	.815	.244	-.282	.751	.164	-.089	.219*	-.129



1. The correlation between fatty acid oxidation capacity (FAO) and the activities of cytochrome oxidase (CytoX) and malate dehydrogenase (MDH) in the different parts of MQF and the whole muscle of 21 mice in EXP III.

Discussion

The largest differences in the properties of red and white tissue samples were recorded in oxidative capacity as expected. Red muscle oxidized fatty acids approx. 4 times faster than white tissue. CytoX and MDH activities were twice as high in red than in white samples. Baldwin *et al.* (1972) have obtained similar results using the superficial, white and deep, red sections of MQF of rat. In rat the differences were, however much more pronounced, fatty acid oxidizing capacity and palmityltransferase activity were approx. seven times, CytoX activity 5 and citrate synthase activity 3 times higher in the red than in the white tissue. The reason for the greater differences in rat is maybe caused by the sample because from rat it is difficult to get the red and the white muscle in a more representative form. In addition, qualitative differences may exist.

The red type of muscle is thus characterized by high oxidative capacity. Petto (1966) has reported that in different kinds of muscles of rabbit the capacities of the pathways for oxidative energy metabolism occur in rather constant ratios. This kind of constancy in enzyme activity ratios probably provides only quantitative differences in the amount of mitochondria in red and white tissues. In the present study in agreement with the results of Baldwin *et al.* (1972) it was shown that the differences in the activities of citric acid cycle and respiratory chain enzymes between red and white muscle were less than in fatty acid oxidation capacity. Baldwin *et al.* (1972) and Beatty *et al.* (1972) have further observed that the difference in FAO is markedly greater than in the capacity to oxidize pyruvate. All these observations suggest that different pathways for oxidative energy production do not occur in exactly constant ratios in the red and white muscle types of mouse and rat. On the contrary they show that the FAO in red tissue is more pronounced. The present results directly give support to the hypothesis that there are also qualitative differences between mitochondria in red and white tissues as observed by Pando and Blanchard (1971). The mitochondria in red muscle are more specialized for fatty acid oxidation.

No clear differences in anaerobic or glycolytic capacities were observed between red and white muscle types. The reason for this might be that in mice the red tissue is fast contracting which is characteristic of rodents (Barnard *et al.* 1971; Baldwin *et al.* 1973). For fast

TABLE IV Correlation coefficients between the measured variables in EXP II. The significance of correlation coefficients. $p < 0.05$, $p < 0.01$ and $p < 0.001$

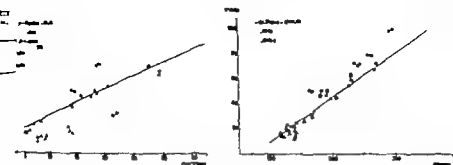
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especially if all measured values from the different muscle samples are accounted (Fig. 3). Correlation coefficients were then statistically highly significant.

Another positive correlation pattern existed between TPDH and LDH activity ($p < 0.001$). In this case correlation coefficients were high also in separate muscle types (Table V). The relationships between TPDH and oxidative variables were with all mostly positive. On the other hand, LDH activity correlated often negatively with oxidative variables. These correlations were statistically significant only in some cases.

TABLE V Correlation coefficients between the measured variables in EXP III in different muscle types separately in control and trained groups and the whole sample. Statistical significance as in Table IV

Muscle group	CytOx/FAO	MDH/FAO	TPDH/FAO	LDH/FAO	MDH/CytOx	TPDH/LDH/CytOx	TPDH/MDH	LDH/MDH	LDH/TPDH
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Controls	.257	.164	-.055	-.062	.402	.179	.249	.094	.324
Trained	.137	.026	.270	.127	.857	.400	.611	.124	.586
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All values									
Controls	.643	.907	.283	-.230	.580*	.230	-.214	.399*	-.083
Trained	.613	.774	.177	-.306	.795	-.24	.003	.035	-.122
Together	.638*	.815	-.44	-.18	.751	.164	-.089	.219*	-.129



1. The correlations between fatty acid oxidation capacity (FAO) and the activities of cytochrome oxidase (CytOx) and malate dehydrogenase (MDH) in the different parts of MQF and the sole muscle of R1 mice in EXP. III

Discussion

Largest differences in the properties of red and white tissue samples were recorded in oxidative capacity as expected. Red muscle oxidized fatty acids approx. 4 times faster than white tissue. CytOx and MDH activities were twice as high in red than in white samples. *Adwin et al.* (1972) have obtained similar results using the superficial, white and deep, red portions of MQF of rat. In rat the differences were, however much more pronounced; fatty acid oxidizing capacity and palmitoyltransferase activity were approx. seven times, CytOx activity 5, and citrate synthase activity 3 times higher in the red than in the white tissue. The reason for the greater differences in rat is maybe caused by the sample because from rat it is possible to get the red and the white muscle in a more representative form. In addition, species differences may exist.

The red type of muscle is thus characterized by high oxidative capacity. *Pette* (1966) has observed that in different kinds of muscles of rabbit the capacities of the pathways for oxidative energy metabolism occur in rather constant ratios. This kind of constancy in enzyme activity ratios probably provides only quantitative differences in the amount of mitochondria in red and white tissues. In the present study in agreement with the results of *Adwin et al.* (1972) it was shown that the differences in the activities of citric acid cycle and respiratory chain enzymes between red and white muscle were less than in fatty acid oxidation capacity. *Baldwin et al.* (1972) and *Beatty et al.* (1972) have further observed that the difference in FAO is markedly greater than in the capacity to oxidize pyruvate. All these observations suggest that different pathways for oxidative energy production do not occur in exactly constant ratios in the red and white muscle types of mouse and rat. On the contrary they show that the FAO in red tissue is more pronounced. The present results indirectly give support to the hypothesis that there are also qualitative differences between the mitochondria in red and white tissues as observed by *Pande and Blanchard* (1971). The mitochondria in red muscle are more specialized for fatty acid oxidation.

No clear differences in anaerobic or glycolytic capacities were observed between red and white muscle types. The reason for this might be that in mice the red tissue is fast contracting which is characteristic of rodents (*Barnard et al.* 1971; *Baldwin et al.* 1973). For fast

contractions, high glycolytic and anaerobic capacities are essential. The differences in these capacities in MQF of rat were slightly higher (Baldwin *et al* 1973) than observed in this study.

It has been repeatedly observed that endurance type of training increases the oxidative capacity of muscle (e.g. Holloszy 1967 Mole 1971 Baldwin *et al* 1972, Gollnick *et al* 1973, Messling *et al* 1974). The activities of enzymes of citric acid cycle, respiratory chain and β -oxidation are increased (Holloszy *et al* 1971 Mole *et al* 1971 Morgan *et al* 1971) as are the capacities to oxidize fatty acids or carbohydrates (Baldwin *et al* 1972, Mole *et al* 1971, Holloszy 1970). In the present study most pronounced increase in FAO was observed in EXP II with F-hybrids. This increase was 36.5% while in EXP I and EXP III the increases were on the average 24%. According to Mole *et al* (1971) the FAO in MQF and posterior cranius-muscles of rat was almost doubled due to endurance training. In the above mentioned study the training of rats was very intensive and that may be the reason for the large increase. The physical inactivity of rats when compared to mice in cage conditions may also influence the magnitude of the increase.

The increase in CytOx and MDH activities was on the average 30%. The increase of FAO in EXP II was higher than in EXP I and EXP III although the increase in CytOx activity in EXP II and EXP III was of the same magnitude. This observation suggests that the training type which provides greater endurance might more strongly adapt β -oxidative capacity. Increased β -oxidative capacity has a sparing effect on muscle glycogen stores as it decreases blood glucose consumption thus preventing fatigue during submaximal loading (Keul *et al* 1972).

Results regarding the effects of endurance training in different muscle types are contradictory. Histochemical studies suggest that the oxidative capacity increases primarily in white fibers (Gollnick *et al* 1973 Zilka *et al* 1973). Biochemical data show that endurance training adapts the oxidative capacity of red muscle more than that of white muscle (Baldwin *et al* 1972). The present results show that the relative increase in oxidative capacity in both muscle types is approximately similar but the absolute change in red tissue is manyfold when compared to white tissue. Both FAO and the activities of CytOx and MDH increased clearly more in the red proximal part of mouse MQF than in the distal white part. The difference in changes was greater in FAO than in CytOx or MDH activities. These observations suggest that either the white muscle tissue is weaker in its adaptive capacity than red muscle or the training affected the white fibers less than the red fibers.

The adaptations caused by training must always be revised against the intensity and duration of training. The training program used by Baldwin *et al* (1973) decreased very strongly the glycogen content in the red part of the thigh muscle of rat whereas it had practically no effects in the white part. Thus it is evident that white muscle cells contracted inactively during the loading. The recruitment order of motor neurons is determined primarily according to the size principle (Granit 1970 Millner Brown *et al* 1972). The small and slowly conductive motor neurons are activated first and they recruit oxidative fibers. When the level of tension increases also large motor units, which are composed of white fibers, are recruited. The increasing tension is achieved at lower tension levels by recruiting new motor units whereas if the tension still increases additional muscular power is mainly produced by higher impulse frequency (Gydikov and Kosarov 1971 Millner Brown *et al* 1972).

2. It is generally accepted that impulse frequency has regulating role also on the catabolic properties of muscle cells (Guth 1968, Cotter *et al.* 1973). Thus red muscle cells showing at higher frequency level during light loading are probably more affected by atrophy adaptive responses. This might explain the higher increase in the oxidative activity in red muscle after training programs used in this study.

The evaluation of the adaptive capacity of different muscle types is difficult on the basis of one experiment series. In the present study a possibility exists that the loading level had activated the motor units in white tissue differently causing a different increase in oxidative capacity of separate motor units. On the other hand, it is not known what is the relationship in the activation between the white surface part or the distal part of the muscle. The effect of endurance training in the white distal part of NMRI-mice (CP III) is rather small when compared to changes observed in rats (Baldwin *et al.* 1972). The reason for this is probably the lighter loading level used in the present study. White muscle fibers are probably rather adaptive, because Cotter *et al.* (1973) have observed that low-level electrical stimulation of white muscle tissue can increase the capillary density and citrate synthase activity close to the level recorded in red tissue already after a few days. However it must be emphasized that this effect provided continuous activation of the fibers.

In the present study it was observed that endurance training increased the oxidative capacity producing capacity in the red and the white muscle in almost constant ratio. Thus homogeneous adaptation of oxidative processes does not change the cell-type distribution but increases the basic level of oxidative metabolism in both fiber types. Endurance training had only slight effects on the glycolytic or anaerobic capacities in the muscle types studied. The adaptive effects of endurance training are thus mainly directed to the oxidative energy metabolism and are qualitatively to a high extent parallel in the red and in the white muscle tissue.

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Difference in Sensitivity of Parotid Glands brought about by Disuse and Overuse

By

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Abstract

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Sensitivity to acetylcholine of the parotid glands in rats maintained on liquid diet (serving as sensitization) for periods of 2 weeks or 3-4 weeks was compared with that of the parotid glands in rats on pelleted diet (serving as desensitization). In the rats on the liquid diet, it was found that the dose needed to produce a perceptible secretion of saliva was smaller, that the secretion started earlier and continued longer time period, and further that the amount of saliva secreted expressed per gland, even in response to submaximal doses of the sialogogue drug, was bigger when compared with the rats on the pelleted diet. These findings are interpreted as signs of higher degree of sensitivity of the glands in rats on the liquid diet than of those in the rats on the pelleted bulk diet.

Rats fed on a liquid diet the parotid glands were found to loose weight, and the acetylcholine synthesizing capacity of the intraglandular cholinergic nerves decreased. These results were thought to be consequences of reduced reflex stimulation of the glands (Ekström 1973). In these experiments the sensitivity of the secretory cells to chemical stimuli was also investigated since decreasing the flow of secretory impulses, for instance by cutting motor fibres of the secretory reflex arc, has been shown to cause a supersensitivity in salivary glands (see Emmelin 1952, 1961 a, 1967). There was some indication that a supersensitivity developed in parotid glands of rats on liquid diet, but when comparisons were made with rats on an ordinary pelleted diet no statistically significant differences appeared. In the present experiments rats on liquid diet were compared with litter mates fed on a bulky diet supposed to increase the reflex stimulation of the glandular cells. Previous experiments have shown that the weight and the acetylcholine forming capacity of parotid glands increase in rats given a cellulose rich diet (Ekström 1974).

Methods

Male rats of Sprague-Dawley strain bred at this Institute were used. The rats were 6-11 months old at the experiments started. They were either given liquid diet, prepared twice daily by mixing 2 parts of liquid with 1 part of powder of standard diet for rats (R 3, Astra-E 06), or pelleted bulk diet made

from the same standard diet but containing 30% cellULOSE instead of the usual 3%. The rats given the liquid diet were litter mates to those given the bulk diet. The diets and water were given *ad libitum*. The rats on the liquid diet were placed in cages with bottoms of wire-netting, instead of bottoms covered by saw shavings, this was done in order to further reduce their opportunity to gnaw. At the start and at the end of the experimental period, the body weights were determined. After an experimental period of 2 weeks or 3-4 weeks, the animals were anesthetized with chloralose (100 mg/kg) i.v. after ether induction. The parotid duct on the right side was exposed near the entrance in the mouth through a skin incision. A cannula connected with a fine glass cannula, which gave 94 drops from 1 ml distilled water. Saliva appearing at the tip of the cannula was collected on filter paper and weighed. To estimate the threshold dose and to obtain maximal secretory responses a series of doses of methacholine chloride (0.05-10 µg/kg) was given. The amount of saliva secreted was expressed in mg per gland or in mg saliva per mg gland weight (dry weight). To estimate the maximal secretory response, as described by Schneyer and Hall (1966) pilocarpine hydrochloride (10 mg/kg) was injected i.p. In this type of expt. drops of saliva were recorded each second over a period of 15 min on a smoked drum with an ordinate writer. The maximal response was expressed as a saliva per min per gland or per min per mg gland weight (dry weight). The two litter mates to be compared, one on the liquid diet and one on the pelleted bulk diet, were always tested consecutively. At the end of the expt. the rats were killed by i.v. injection of air and the parotid glands, which had been cannulated for 1-2 secretory response was carefully dissected out, cleaned and weighed before (wet weight) and after being dried to 105-110°C for 48 h (dry weight). From rats studied over a period of 3-4 weeks the parotid glands on the left side which was not cannulated were also removed, cleaned and prepared for histology of acetylcholine acetyltransferase activity using the method of Catherine Hebb (Nordenfjeld 1967). The enzyme activity was expressed in µg acetylcholine chloride formed per hour per pool of glands (total wet weight) and in µg acetylcholine chloride formed per hour per g acetone powder (concentration). Further details are given in the text.

For statistical analysis Student's *t* test was used, paired comparisons were made between the rats on the liquid diet and its litter mate given the pelleted bulk diet. *P*-values less than 0.05 are considered significant.

Results

Body weights. At the start of the expts. there were no differences in the weights between the rats to be given the liquid diet and those to be given the pelleted bulk diet. In the series of 2 weeks the body weights of the rats on the liquid diet increased ($p < 0.001$) from (mean \pm S.E.) 350 ± 9 to 361 ± 7 g ($n=20$), i.e. by about 3%, while that of the rats on the pelleted bulk diet remained unchanged (from 353 ± 8 to 354 ± 7 g, $n=20$). In the series of 3-4 weeks there was also an increase ($p < 0.05$) in body weights of the rats on the liquid diet from 371 ± 4 to 386 ± 5 g ($n=17$), i.e. by about 2%, while the weights of the rats on the pelleted bulk diet did not change (from 377 ± 5 to 380 ± 5 g, $n=17$).

Gland weights. As shown in Table I the wet weights of the parotid glands in the rats on the liquid diet, both in the series of 2 weeks and in the series of 3-4 weeks, were about 50% of those in the rats on the pelleted bulk diet. The dry weights were similarly affected.

TABLE I Gland weights (wet and dry) of parotid glands in rats given liquid diet and in rats given pelleted bulk diet for 2 weeks and 3-4 weeks. Values are mean \pm S.E. in brackets are numbers of observations.

		Wet weights (mg)	Liquid/ pelleted ()	Dry weights (mg)	Liquid/ pelleted ()
2 weeks	Liquid diet	90.6 \pm 3.1 (20)	47.7 \pm 2.2 (20) $p < 0.001$	6.7 \pm 0.9 (20)	49.7 \pm 3.0 (20) $p < 0.001$
	Pelleted bulk diet	194.2 \pm 7.9 (20)		55.5 \pm 2.4 (20)	
3-4 weeks	Liquid diet	99.7 \pm 5.4 (17)	48.2 \pm 1.1 (17) $p < 0.001$	30.9 \pm 1.9 (17)	46.3 \pm 3.1 (17) $p < 0.001$
	Pelleted bulk diet	218.4 \pm 9.0 (17)		67.6 \pm 2.9 (17)	

II The secretory responses of parotid glands in μg saliva after 2, 5 and 10 $\mu\text{g}/\text{kg}$ l. of methacholine in rats given liquid diet and in rats given pelleted bulk diet for 2 and 3-4 weeks. Values are mean \pm S.E. In brackets are numbers of observations

	2 $\mu\text{g}/\text{kg}$	5 $\mu\text{g}/\text{kg}$	10 $\mu\text{g}/\text{kg}$
2 weeks Liquid diet	5.7 \pm 0.9 (15)	9.5 \pm 0.9 (15)	17.9 \pm 1.5 (15)
Pelleted bulk diet	4.6 \pm 1.0 (15)	10.8 \pm 1.0 (15)	21.2 \pm 1.8 (15)
3-4 weeks Liquid diet	2.7 \pm 0.4 (10)	6.3 \pm 0.5 (10)	14.7 \pm 1.5 (10)
Pelleted bulk diet	4.2 \pm 0.5 (10)	10.0 \pm 0.8 (10)	24.2 \pm 3.4 (10)

Acetylcholinesterase activity The amount of acetylcholine formed during 1 h by a pool of 11 parotid glands from rats fed on the liquid diet over the period of 3-4 weeks was 3 μg , while the pool of 11 glands from rats fed on the pelleted bulk diet over the same period of time formed 317.5 μg , i.e. the total enzyme activity was about 30% less in the parotid glands of the rats on the liquid diet. As to the enzyme concentration (per g acetone wet wt) it was 841 μg acetylcholine in the glands of the rats on the liquid diet and 560 μg acetylcholine in the rats on the pelleted bulk diet. The fact that the enzyme concentration was lower in the rats on the liquid diet is attributed to the pronounced difference in gland weight between the two groups of animals.

Secretory threshold doses. When the two litter mates were compared, one given the liquid diet and the other the pelleted bulk diet, it was found that the amount of methacholine had to evoke a just perceptible secretion of saliva from the parotid gland was the same whether low for the rat on the liquid diet. In the series of 2 weeks the mean threshold dose was 0.34 \pm 0.08 $\mu\text{g}/\text{kg}$ for 15 rats on the liquid diet and for the corresponding 15 rats on the pelleted bulk diet 0.49 \pm 0.09 $\mu\text{g}/\text{kg}$. In the series of 3-4 weeks the dose was 0.80 \pm 0.08 $\mu\text{g}/\text{kg}$ for 10 rats given the liquid diet and 1.0 \pm 0.0 $\mu\text{g}/\text{kg}$ for the corresponding 10 rats on the pelleted bulk diet. On both occasions the difference between the two groups of rats was significant at the $p < 0.05$ level.

Secretory responses to some standard doses. The amount of saliva secreted in response to 5 and 10 $\mu\text{g}/\text{kg}$ of methacholine is shown in Table II and III. In Table III the saliva is related to the gland weight. In the series of 2 weeks the responses of the glands in the rats on the liquid diet were about the same as those of the rats fed on the pelleted bulk diet

at III The secretory response of parotid glands in μg saliva per μg mean after 2, 5 and 10 $\mu\text{g}/\text{kg}$ l. of methacholine in rats given liquid diet and in rats given pelleted bulk diet for 2 weeks and 3-4 weeks. Values are mean \pm S.E. In brackets are numbers of observations

	2 $\mu\text{g}/\text{kg}$	5 $\mu\text{g}/\text{kg}$	10 $\mu\text{g}/\text{kg}$
2 weeks Liquid diet	0.2182 \pm 0.0330 (15)	0.3617 \pm 0.0378 (15)	0.6221 \pm 0.0649 (15)
Pelleted bulk diet	0.1232 \pm 0.0180 (15)	0.2005 \pm 0.0208 (15)	0.3904 \pm 0.0355 (15)
3-4 weeks Liquid diet	0.0929 \pm 0.0156 (10)	0.2161 \pm 0.0256 (10)	0.4934 \pm 0.0534 (10)
Pelleted bulk diet	0.0623 \pm 0.0065 (10)	0.1500 \pm 0.0134 (10)	0.3408 \pm 0.0375 (10)

from the same standard diet but containing 30% cellulose instead of the usual 3%, the rats given this diet were litter mates to those given the bulk diet. The diets and water were given *ad libitum*. The rats on the liquid diet were placed in cages with bottoms of wire-netting, instead of bottoms covered by wood shavings; this was done in order to further reduce their opportunity to gnaw. At the start and at the end of the experimental period, the body-weights were determined. After an experimental period of 2 weeks of 3-4 weeks, the animals were anaesthetized with chloralose (100 mg/kg) i.v. after ether sedation. The parotid duct on the right side was exposed near the entrance in the mouth through a skin incision and cannulated with a fine glass cannula, which gave 94 drops from 1 ml distilled water. Each day the tip of the cannula was collected on filter paper and weighed. To estimate the threshold dose and the maximal secretory responses a series of doses of methacholine chloride (0.05-10 µg/kg) was given. The amount of saliva secreted was expressed in mg per gland or in mg saliva per mg gland (dry weight). To estimate the maximal secretory response, as described by Schneyer and Hall (1966) pilocarpine hydrochloride (10 mg/kg) was injected i.p. In this type of expt. drops of saliva were recorded each over a period of 15 min on a smoked drum with an ordinate writer. The maximal response was expressed as mg saliva per min per gland or per min per mg gland weight (dry weight). The two litter mates to be compared, one on the liquid diet and one on the pelleted bulk diet, were always tested consecutively. At the end of the expt. the rats were killed by i.v. injection of air and the parotid gland which had been removed by the secretory response was carefully dissected out, cleaned and weighed before (wet weight) and after heated to 105-110°C for 48 h (dry weight). From rats studied over a period of 3-4 weeks the parotid glands on the left side which was not cannulated were also removed, cleaned and prepared for bioassay of the choline acetyltransferase activity using the method of Catherine Hebb (Nordenskiöld 1963). The red activity was expressed as µg acetylcholine chloride formed per hour per pool of glands (total score) and as µg acetylcholine chloride formed per hour per g acetone powder (concentration). Further details are given in the text.

For statistical analysis Student's *t*-test was used. Paired comparisons were made between the rats on the liquid diet and litter mates given the pelleted bulk diet. *P*-values less than 0.05 are considered significant.

Results

Body weights At the start of the expts. there were no differences in the weights between the rats to be given the liquid diet and those to be given the pelleted bulk diet. In the series of 2 weeks the body weights of the rats on the liquid diet increased ($p < 0.001$) from (mean \pm S.E.) 350 ± 9 to 361 ± 7 g ($n = 20$), i.e. by about 3%, while that of the rats on the pelleted bulk diet remained unchanged (from 353 ± 8 to 354 ± 7 g, $n = 20$). In the series of 3-4 weeks there was also an increase ($p < 0.05$) in body weights of the rats on the liquid diet from 377 ± 5 to 386 ± 5 g ($n = 17$), i.e. by about 2%, while the weights of the rats on the pelleted bulk diet did not change (from 377 ± 5 to 380 ± 5 g, $n = 17$).

Gland weights As shown in Table I the wet weights of the parotid glands in the rats on the liquid diet, both in the series of 2 weeks and in the series of 3-4 weeks, were about 60% of those in the rats on the pelleted bulk diet. The dry weights were similarly affected.

TABLE I. Gland weights (wet and dry) of parotid glands in rats given liquid diet and in rats given pelleted bulk diet for 2 weeks and 3-4 weeks. Values are mean \pm S.E. in brackets are numbers of observations.

		Wet weights (mg)	Liquid/ pelleted (%)	Dry weights (mg)	Liquid/ pelleted (%)
2 weeks	Liquid diet	90.6 ± 3.1 (20)	47.7 ± 2.2 (70) $p < 0.001$	6.7 ± 0.9 (70)	49.2 ± 3.0 (70) $p < 0.001$
	Pelleted bulk diet	194.2 ± 7.9 (20)		35.5 ± 2.4 (70)	
3-4 weeks	Liquid diet	99.7 ± 5.4 (17)	48.2 ± 1.1 (17) $p < 0.001$	30.9 ± 1.9 (17)	46.3 ± 3.1 (17) $p < 0.001$
	Pelleted bulk diet	184 ± 9.2 (17)		67.6 ± 2.9 (17)	



LIQUID DIET

PELLETED BULK DIET

The maximal secretory response to pilocarpine, 10 mg/kg i.p. of the parotid gland of rat maintained on liquid diet and of its litter mates maintained on pelleted bulk diet; the rats belonged to the series of 3-4 weeks. Records from above: ink marks, drops of saliva, signal, marking the start of the injection of pilocarpine and the onset of secretion, salivary flow rate recorded with an ordinate writer. The time of the secretion was in the rat on the liquid diet 25.1 and in the rat on the pelleted bulk diet 30.7 after the injection of pilocarpine.

on liquid diet. In Fig. 1 the recordings from two litter mates, one on liquid diet and the other on pelleted bulk diet, are shown after the injection of pilocarpine; in these recordings the time of onset of secretion was also marked.

When expressed in mg saliva per min and mg tissue, both series showed a bigger maximal response ($p < 0.001$) in the rats on the liquid diet. The figures in the series of 2 weeks were 0.10 (liquid diet) and 0.63 ± 0.04 (pelleted bulk diet), and in the series of 3-4 weeks 1 ± 0.09 and 0.76 ± 0.05 respectively.

Discussion

As the body-weights of the rats are considered it appears that the animals endured the diet well. As could be expected from the previous findings (Ekström 1973-1974) the weights, particularly but also the acetylcholine forming capacity of the parotid glands were lower in the rats maintained on the liquid diet than in those on the pelleted bulk diet. In studies on sensitivity of salivary glands comparisons have usually been made between the experimental gland and its fellow gland in one and the same animal or repeated observations have been made over long periods of time on a gland (Emmellin 1964). A technique allowing repeated examinations of the parotid glands in rats has not been developed yet, except to reduce the number of variables was in the present study to make comparisons between litter mates and further to examine the two litter mates on the same occasion. The degree of sensitivity of a gland to chemical stimuli may be expressed in terms of threshold dose, time of onset and duration of secretion, and volume of saliva secreted in response to a submaximal dose, as pointed out by Emmellin (1952). Sensitization is known to be most marked after postganglionic section of the main secretory nerve, the parasympathetic including not only the action on the gland cells of that fraction of the transmitter set free

when not related to the weights. In the series of 3-4 weeks the amount of saliva at all the doses, less in the animals on the liquid diet. When the amount of saliva was related to the gland weight it can be seen that in the series of 2 weeks the liquid diet secreted a bigger amount of saliva than those on the pelleted bulk diet doses tested. In the series of 3-4 weeks the amount of saliva was the same in the at the dose of 2 $\mu\text{g/kg}$ and bigger in the rats on the liquid diet at the two larger III shows also that the differences in the amount of saliva secreted per gland bigger in the series of 2 weeks than in the series of 3-4 weeks, e.g. when co figures at the dose of 5 $\mu\text{g/kg}$ the amount of saliva secreted per gland weight in the the liquid diet was in percent of that in the rats on the pelleted bulk diet about 1 series of 2 weeks and in the series of 3-4 weeks about 145

Onset of secretion It was noticed that the animals on the liquid diet seemed to respond more promptly to an injection of methacholine than those on the other type of diet, i.e. when the time that elapsed between the beginning of the i.v. injection of 5 $\mu\text{g/kg}$ of the substance and the start of the secretion (i.e. just when the meniscus of saliva began to move) was measured. It was found that both in the series of 2 weeks and in the series of 3-4 weeks the rats on the liquid diet responded about 1 s earlier ($p < 0.01$). In the series of 2 weeks the figures were 6.2 ± 0.4 s ($n=9$) for the rats on the liquid diet and 7.4 ± 0.4 s ($n=9$) for the rats on the pelleted bulk diet and in the series of 3-4 weeks 6.7 ± 0.1 s ($n=8$) and 7.7 ± 0.1 s ($n=8$) respectively.

Duration of secretion A striking feature was that the secretion of the rats on the liquid diet continued over a longer period of time than that of the rats on the pelleted bulk diet. The time of secretion was determined using the dose of 5 $\mu\text{g/kg}$ methacholine. In this type of expt. care was taken to remove saliva appearing at the tip of the cannula frequently in order to make it easier to observe the end of the secretion. The duration of the secretion was about twice as long ($p < 0.001$) in the animals on the liquid diet. In the series of 2 weeks the mean duration was 128 ± 8 s in 10 rats on the liquid diet and 66 ± 4 s in 10 rats on the pelleted bulk diet. In the series of 3-4 weeks the figures were 114 ± 8 s ($n=9$) and 69 ± 3 s ($n=9$) respectively.

Maximal secretory response In both the series of 2 weeks and that of 3-4 weeks the maximal secretory response to pilocarpine of the parotid glands, expressed in μg saliva per min was found to be less ($p < 0.02$) in the rats on the liquid diet than in those on the pelleted bulk diet. In the series of 2 weeks the mean response was, in 5 rats given the liquid diet 26.1 ± 1.8 and in the corresponding 5 rats on the pelleted bulk diet 34.2 ± 1.1 i.e. the response after the liquid regime was in percent of that after the pelleted bulk regime 76.6 ± 5.4 ($n=5$). In the series of 3-4 weeks the mean response in 6 rats on the liquid diet was 30.9 ± 2.1 and in the 6 rats on the pelleted bulk diet 41.8 ± 2.5 the percentage figure obtained was very similar to that of the series of 2 weeks, being 75.1 ± 6.1 ($n=6$). It appeared from the traces in this type of expt. that the maximal secretory response was reached earlier in the rats on the liquid diet and further that the rate of secretion in these rats was not maintained at a high level for such a long time as in those on the pelleted bulk diet. Although the i.p. injection of pilocarpine could not be as standardized as the i.v. injection of methacholine, it may be mentioned that the onset of secretion to pilocarpine was earlier in the rats on the liquid diet.

serotid glands of the rats on the liquid diet had a maximal secretory capacity that was 1-25 percent less than that of the rats on the pelleted bulk diet both in the series of 2-3 and in the series of 3-4 weeks. The maximal secretory capacity of a salivary gland is known to be reduced by parasympathetic denervation or decentralization, and this is probably connected to the glandular atrophy after these operations (Emmelin, Malm and Jodal 1960).

It seems reasonable to conclude from the present expts. that a difference in sensitivity of serotid glands in rats supplied with an intact innervation can be brought about by the (1) an experimental set up favouring acclimatization of the gland cells (liquid diet) on one hand and decentralization (pelleted bulk diet) on the other hand as to decentralization, it is known that prolonged treatment with atropine-like drugs can depress the sensitivity of the gland cells (Emmelin 1952).

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by nerve impulses but also that fraction of the transmitter continuously leaking from the nerve endings, see Emmelin 1967). It is less marked after section of the preganglionic nerve and even less after cutting afferent nerves from the tongue (Emmelin 1961 b). From our observations it appears to follow that the difference in sensitivity that may be expected to be at hand in the present experiments using animals with intact nervous pathways is small. Nevertheless, the present findings of lower threshold dose to methacholine, and of earlier start and longer duration of secretion in response to this substance in the rats given the liquid diet when compared with the rats on the pelleted bulk diet seem to indicate that the parotid glands of the rats on the liquid diet had a higher degree of sensitivity than those of the rats on the pelleted bulk diet. Such an idea is further supported by the results of the experiments in which pilocarpine was used: the secretion both started (at least in most cases) and reached the maximal flow rate earlier in the rats on the liquid diet than in the rats on the pelleted bulk diet. The glands of the rats on the liquid diet appeared to be more exhaustible, and they only maintained a high flow rate for a short time: this is a phenomenon seen in connection with sensitization (Emmelin and Muren 1951).

Since the difference in threshold was relatively small it is of interest to note that in the rat treated over a period of time with a ganglion blocking substance the dose of methacholine needed to evoke secretion of saliva from the parotids was half of that needed for the glands in untreated animals (Ekström and Lindmark, to be published). The pathway of the preganglionic parasympathetic nerves of the parotid glands in this species is unknown and therefore these nerves could not be cut. In the cat the parasympathetically denervated parotid gland had a threshold dose to acetylcholine that was also about half of that of the contralateral gland (Strömblad 1955).

The volume or amount of saliva secreted in response to some standard doses of methacholine was the same in the two groups of animals in the series of 2 weeks, and in the series of 3-4 weeks it was even smaller in the rats on the liquid diet than in those on the pelleted bulk diet. For comparisons it may be mentioned that after prolonged ganglion blockade the amount of saliva secreted to a dose of 5 g/kg methacholine was about 20 per cent more than from the parotid glands in untreated rats (Ekström and Lindmark, to be published) whereas after a postganglionic parasympathetic denervation the amount of saliva secreted was about 3 times that of the contralateral gland in response to the same dose of methacholine (Alm and Ekström 1976). When the amount of saliva secreted to the submaximal doses of methacholine is related to the gland weight it is found that the gland of the rat on the liquid diet secreted more than the gland of the rat on the pelleted bulk diet, and this applies both for the series of 2 weeks and the series of 3-4 weeks. Because of the very big difference in gland weight between the rats on the liquid diet and those on the pelleted bulk diet it does not seem relevant to use the response to submaximal doses of methacholine as a parameter of sensitivity without relating the response to the gland weight. The higher figures obtained from the rats on the liquid diet, when the comparison is based on the gland weights, are interpreted as another sign of a higher degree of sensitivity of the glands of these animals compared with those in the rats on the pelleted bulk diet. That the weight is of importance for the amount of saliva secreted is shown by the results of the experiment in which the maximal secretory response to an i.p. injection of pilocarpine was determined.

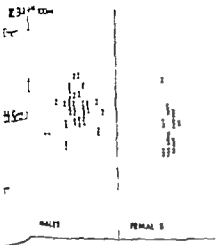


Fig. 1

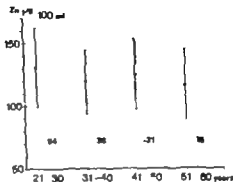


Fig. 2

1. Serum zinc levels of male and female blood donors from Oslo. Lines indicate mean levels \pm S.D.
2. Serum zinc levels in men at different ages. Mean \pm S.D.

men of age 22-60 years were recalled for further examinations because of their serum zinc levels were higher than the local mean in 19 men and lower than the mean in 21 men. Only 3 of them improved disease. Subject number 24 (Table I) had an atherosclerosis, number 23 heart and number 36 (Table II) an arterial hypertension. They used regularly drugs according to their condition. Blood samples of these 40 men were examined on a Technicon Autoanalyzer as rough

screen of their condition. Blood samples were drawn at 8 a.m. after an overnight fast. Clotted samples were centrifuged and the serum was used. For hormone determinations serum was conserved in -20°C till the assays were made.

The zinc determinations were performed using Perkin-Elmer Model 303 atomic absorption spectrophotometer. Standard solutions were prepared using stock solutions (Merck, Germany). The difference in viscosity between the serum and the aqueous standard solutions was avoided by the addition of a known volume of water (Hackley *et al.* 1968). Standard solutions contained 154 μg of Zn and Cd. The samples were undiluted, since optimal sensitivity could be obtained in the recorder at this range. Sources of contamination (rubber stoppers etc.) were excluded as far as possible and deionized serum was used.

Testosterone stimulating hormone (FSH) and serum luteinizing hormone (LH) were determined by double antibody radioimmunoassay (Reiter *et al.* 1973). The second antibody was fixed on a solid support according to the method of Wide *et al.* (1969). Kits by CEA-IRE-SORIN were used and IER 907 as used as reference preparation. Radioimmunoassay of serum testosterone was performed after extraction of 30 μl of serum in diethyl ether (ad 3.0 ml) as described by Dufon *et al.* 1972. The statistical treatment of the data regression analysis (Spearman correlation coefficient) and

Results

Mean serum zinc and height. The mean serum zinc level \pm S.D. of the 154 male blood donors was $123.84 \pm 28.49 \mu\text{g}/100 \text{ ml}$ and that of the 95 females $111.36 \pm 23.40 \mu\text{g}/100 \text{ ml}$. The difference between the men and women was highly significant ($p < 0.001$). Fig. 2 shows mean serum

Serum Testosterone Compared with Serum Zinc in Man

By

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Abstract

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Serum zinc was measured in 154 male and 95 female blood donors. Men had higher serum zinc levels than women, the difference being statistically significant ($p < 0.001$). The correlations between serum zinc, serum testosterone, serum zinc and serum FSH and serum zinc and serum LH were studied in 61 aged 38-60 years and a statistically significant positive correlation was found only between serum zinc and serum testosterone in men of age 36-60 years ($p < 0.005$). These results do not support the theory of slight zinc deficiency primarily effects pituitary gonadotropins but indicate that it would rather on testicular level.

There are very few observations of zinc deficiency in man. External features evidently to zinc deficiency described by Prasad in a group of Iranian adult males were growth retardation and marked hypogonadism (Prasad *et al* 1961), and Prasad also found similar cases in Egypt (Prasad *et al* 1963). Therapy with zinc resulted in increased size of external genitalia and the appearance of secondary sex characteristics in addition to increase in height.

Zinc deficiency presents an extreme condition. It was therefore interesting to inquire whether there is any relationship between zinc and pituitary-gonadal axis in normal man. It seemed reasonable to estimate the mean level of serum zinc in the local population as the values stated in the literature differ (Kahn *et al* 1965, Prasad *et al* 1965). For comparison the serum zinc level was also measured in women. Men whose zinc values deviated from the mean in either direction were selected for further investigation, including determination of serum testosterone, FSH and LH.

Material and Methods

Subjects. Serum zinc levels were measured in 154 male and 95 female voluntary blood donors who spontaneously to the Blood Transfusion Service in Oulu during one summer month in 1975. These subjects were presumed to represent the healthy population in this town. Forms with questions about the personal data, weight and height, diseases and use of drugs were sent to the persons subjected.

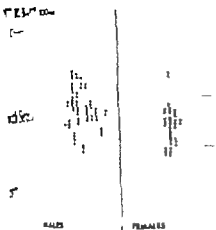


Fig. 1

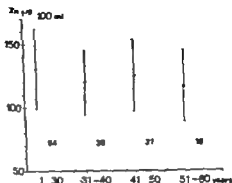


Fig. 2

Serum zinc levels of male and female blood donors from Oslo. Lines indicate mean levels \pm S.D.

Serum zinc levels in men at different ages. Mean \pm S.D.

men of age 23-40 years were recalled for further examinations because of their serum zinc levels were higher than the local mean in 19 men and lower than the mean in 21 men. Only 3 of them diagnosed disease. Subject number 24 (Table II) had an arthritic deformity, number 25 heart and number 36 (Table III) an arterial hypertension. They used regularly drugs according to their illness. Blood samples of these 40 men were examined using a Technicon Autoanalyzer as a rough test of their condition.

Blood samples were drawn at 8 a.m. after an overnight fast. Clotted samples were centrifuged and the serum was used. For hormone determinations serum was conserved at -20°C till the assays were made.

The zinc determinations are performed using Perkin-Elmer Model 303 atomic absorption spectrophotometer. Standard solutions were prepared using stock solutions (Merck, Germany). The difference in viscosity between the serum and the aqueous standard solutions was avoided by adding solution instead of water (Hackley *et al.* 1966). Standard solutions contained 154 μg of Zn and Cd. The samples were analyzed, since optimal sensitivity could be obtained in the recorder also in this range. Sources of contamination (rubber stoppers etc.) are excluded as far as possible and homolyzed serum was used.

Testosterone and luteinizing hormone (LH) are determined by double antibody radioimmunoassay (Rouser *et al.* 1973). The second antibody was fixed on beads according to the method of Wide *et al.* (1969). Kits by CEA-IRE SOGIN were used. LH 947 was used as reference preparation. Radioimmunoassay of serum testosterone was performed by the reaction of 30 μl of serum in diethyl ether (ad 3.0 ml) as described by Dufau *et al.* 1972. The statistical treatment of the data regression analysis (Spearman correlation coefficient) and t -test were used.

Results

Sex, age and height. The mean serum zinc level \pm S.D. of the 154 male blood donors was $123.84 \pm 28.49 \mu\text{g}/100 \text{ ml}$ and that of the 95 females $111.36 \pm 23.40 \mu\text{g}/100 \text{ ml}$. The difference between the men and women was highly significant ($p < 0.001$). Fig. 1 shows mean serum

TABLE I Serum zinc levels, hormone levels and other data of 15 men aged 28-35 years

Subject No	Age in years	Height cm	Weight kg	Zinc μ g/100 ml	Testosterone ng/ml	FSH ng/ml LER 907	LH ng/ml LER 907	Occupation
1	28	177	87	154	10	63	81	Postman
2	28	—	—	160.5	5.5	163	102	Bus driver
3	33	167	62	131	6.0	170	31	Service station keeper
4	33	173	84	145.5	6.4	97	191	Driftman
5	33	176	73	220	2.1	170	92	Engineer
6	34	167	74	148	4.2	99	60	Technician
7	35	185	90	127.5	5.4	170	81	Baker
n, 7								
Mean	32.00	174.16	78.33	155.21	5.64	125.28	91.14	
\pm S.D.	± 2.82	± 6.82	± 10.55	± 30.89	± 2.39	± 43.06	± 49.80	
8	30	186	84	73.5	16	68	56	Geologist
9	30	—	—	99.5	6.7	135	63	MD
10	30	183	70	10	5.5	156	42	University Laboratory
11	31	—	—	93.5	6.5	106	83	—
12	3	175	70	101	6.3	149	42	Correlator
13	34	171	75	93	3.9	9	88	Engineer
14	35	171	81	85	7	117	63	Printer
15	35	—	—	113	13	110	50	Policeman
n, 8								
Mean	32.13	177.20	76.00	95.06	8.11	116.12	58.37	
\pm S.D.	± 2.23	± 6.94	± 6.36	± 11.93	± 4.13	± 29.45	± 14.89	

zinc levels (\pm S.D.) in men at different ages. There was a slight tendency to lower zinc levels in men after the age of 30 years, but the difference was not significant at the 0.05 level ($p > 0.05$). There were no statistically significant differences in the height between the men with low serum zinc and the men with high serum zinc (Tables I, III).

Serum zinc, testosterone and gonadotropins The individual values and mean \pm S.D. for the different groups and other relevant data are given in Table II. In men aged 36-60 years there was a significant positive correlation between serum zinc and serum testosterone ($p < 0.005$) (Fig. 3). In men of 28-35 years there was no significant correlation ($R = -0.43$, $p > 0.10$) between zinc and testosterone. No correlation was established between serum zinc and serum FSH or LH.

Discussion

Earlier literature contains controversial data as to sex difference in serum zinc concentrations (Davies *et al* 1968; Halsted *et al* 1970; Lindeman *et al* 1971). According to the results, men have significantly higher serum zinc levels than women, although the difference is not great, which would explain the controversy especially in studies with limited number of cases. The existence of sex difference and the age related decrease in serum zinc, if being convincingly proved by Lindeman *et al*, suggest that serum zinc and reproductive functions might be in some relation. Also the high concentrations of zinc in the

of Testosterone and LH serum zinc levels, hormone levels and other data of 25 men aged 36-60 years.

Age in years	Height cm	Weight kg	Zinc $\mu\text{g}/100\text{ ml}$	Testosterone ng/ml	FSH ng/ml LER 907	LH ng/ml LER 907	Occupation
36	175	70	170	6.2	135	77	Agricultural agent
48	172	80	156	16	192	60	Factory worker
50	178	93	157	6.9	92	83	Foreman
51	171	73	171.5	16	78	21	Fabricator
46	176	76	216	8	35	46	Engineer
46	170	95	191	15	142	120	Truck driver
47	168	73	124	4.0	99	21	Truck driver
47	—	—	127.5	3.3	156	53	—
49	162	85	157.5	5.1	142	88	Pensionary
50	173	77	168	7.0	106	16	Pensionary
58	175	89	139	4.7	248	99	Engineer
60	164	90	140	3.0	227	77	Factory worker
47.50	171.36	80.09	159.36	8.10	137.66	61.75	
± 7.85	± 5.14	± 9.28	± 27.61	± 4.76	± 61.91	± 33.58	

Age in years	Height cm	Weight kg	Zinc $\mu\text{g}/100\text{ ml}$	Testosterone ng/ml	FSH g/ml LER 907	LH g/ml LER 907	Occupation
37	173	75	89.5	6.9	106	26	Forester
37	171	73	99.5	3.7	54	35	Factory worker
38	—	—	99.5	6.5	215	42	Bus driver
39	177	76	103	4.8	270	38	Railroad man
40	—	—	102	6.4	270	113	—
41	180	87	99.5	10	213	116	Truck driver
42	172	78	90.5	3.3	71	53	Inspector
43	172	85	102	4.1	99	35	Truck driver
45	180	92	92.5	6.1	85	38	Agent
49	172	78	86	4.0	170	93	Construction worker
51	178	100	90.5	3.7	120	70	Railroad man
51	179.5	71	95	3.7	135	56	Teacher
56	165	68	88	3.2	128	31	Forester
43.76	175.40	80.36	95.03	5.10	148.76	57.30	
4.16	± 6.54	± 9.60	± 6.20	± 1.97	± 72.42	± 31.06	

especially in males, support this possibility (Bertrand *et al* 1971 Mawson *et al* 1955).
 Coble and his coworkers have found that in severely zinc-deficient Egyptian there is a delayed puberty with low plasma testosterone and LH (Coble *et al* 1971).
 He concluded that zinc deficiency primarily disturbs the pituitary function. However
 the present study serum FSH or serum LH did not show remarkable fluctuations in
 men with different age or serum zinc levels. Therefore, it is not evident that in nutritionally
 normal adults zinc could interfere with reproductive functions primarily via pituitary

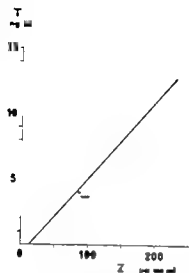


Fig. 3 Correlation between serum zinc and serum testosterone in men aged 36-60 years. Testosterone = $0.06 Z - 0.5$, $R = 0.51$, $p < 0.005$

Also our recent results (to be published) on zinc-deficient rats show a nearly normal fertility function in spite of a disturbed testicular function.

At the moment it is not possible to explain why the consequences of low serum zinc are uniform. Zinc might be a structural part of proteins involved in the synthesis and transport of testosterone. It is known to be the active part of several enzymes, the most important metallo-enzymes of zinc being alcohol dehydrogenases and other dehydrogenases (Hartoma *et al.* 1969). Zinc could also interfere with the hepatic metabolism of testosterone. It interferes with hepatic drug metabolism in animals (Becking *et al.* 1970) and probably in man too (Hartoma *et al.* 1977). In fact, in our previous study a prolonged effect of oestrogen was found in zinc-deficient female rats (Hartoma 1975). Altogether there is circumstantial evidence that zinc deficiency primarily has an inhibitory effect on the steroid synthesis on one side and metabolism on the other. Hence, variable testosterone levels would be expectable in subjects with differing testicular and hepatic functions.

I wish to thank Professors Väinö Veljola and Jorma Sohlo for their kind permission to use the atomic absorption spectrophotometer at the Department of Process Engineering, University of Oulu. Thanks are due to Miss Tyne Mänttä and Mr. Valter Haverinen for their technical assistance. I also thank the staff of the Blood Transfusion Service in Oulu for collecting the samples.

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Regional Brain Uptake of Noradrenaline Following Mechanical or Osmotic Opening of the Blood Brain Barrier

By

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Abstract

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The passage of noradrenaline from the cerebrovascular circulation into the brain (from cells and parenchyma) was studied quantitatively and regionally by modification of Oldendorf's technique for determination of brain uptake index. Using ^{14}C -ethanol as the highly diffusible internal standard, index for noradrenaline varied between 2.7 and 4.5 in different regions, confirming the poor passage of this neurotransmitter. The barrier was impaired transiently as evidenced by E and blue coronal through osmotic opening by internal carotid injection of a hyperosmolar urea solution or vascular disruption by short lasting elevation of the intracarotid hydrostatic pressure. This resulted in a 3-4 increase in the passage of noradrenaline from the circulation into the brain.

There is a common opinion based on the limited number of reports so far available, that the blood-brain barrier almost totally prevents the entry of many amines from the blood into the brain substance proper. Additional support for this opinion has come from behavioral and cerebral blood flow studies where little or no effect is obtained following the systemic administration of amines (Purves 1972, Lassen 1974, Edvinsson and Mackenzie 1977). Oldendorf (1971) has demonstrated that both amines and amino acids in fact are extracted from the circulation in the brain but to a highly varying extent. Thus, many of the amines, such as the neurotransmitters, have a "brain uptake index" (obtained during extraction from the blood) which is only in the range of 2.5 to 4.5 when compared to uptake of a highly diffusible standard. Essential amino acids, on the other hand, are readily taken up from the brain circulation, probably with the aid of active uptake mechanisms (Bartistini, Grynbaum and Lajtha 1971).

A modified procedure for estimating the brain uptake index was utilized to study regional variations in the uptake of tritiated noradrenaline from the blood to the brain. The ability of the amine to penetrate the blood-brain barrier was also examined under two experimental

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are known to open the structural barrier by intracarotid infusion of hyperosmotic opening) and by intracarotid injection of blood at high pressure disruption). The impairment of the barrier function and the reversibility of the visualized by administration of Evans blue into the circulation.

Methods

It was carried out on 52 adult male Sprague-Dawley rats, weighing 300-350 g and fed by a standard diet *ad lib*. The animals were initially anesthetized in a glass jar containing 2-3 soaked charcoal as insecticide and anesthesia was maintained with a mixture of 70% N_2O . The body temperature was maintained around 37°C as measured by rectal thermometer. Cannulae were placed in the femoral arteries and veins on both sides. One of the femoral arterial was used for continuous recording of mean arterial blood pressure and the other artery was also sampling of blood for measurement of blood gases ($PaCO_2$ and PaO_2) which, together quantified in 100 μ l samples with MUK II blood gas analyzer (Radiometer Copenhagen), were maintained at normocapnia ($PaCO_2$ between 35 and 40 mmHg). The main trunk of the carotid artery was cannulated contrilaterally with the catheter opening placed within 1 mm from bifurcation, and the retrograde branches of the ipsilateral external carotid artery. In order to facilitate cannulation, the external carotid artery was exposed by dividing the skin into the thyroid bone, back over the distal portion of the artery. Injections through the external carotid artery were then carried into the internal carotid artery directly to the

ing of the blood-brain barrier

4. 1 ml of Krebs-Ringer buffer solution (pH 7.4, 37°C) containing 2 M sucrose was infused in the cannulated external carotid artery to reach the brain. 10 of the animals had received 20 min beforehand (1 ml of 1.5 M sucrose in Krebs-Ringer buffer). This substance blocks synapses (Friedman and Johnson 1969) and does not, therefore, penetrate the blood-brain barrier. After further 10 min the animals were killed for microscopic and microscopic examination (see below). In 5 of the animals, the infusion of 2 M sucrose was followed by bolus containing mixture of radioactive noradrenaline and ethanol (see further 5 animals (used as controls with an intact blood-brain barrier), 1 ml Krebs-Ringer solution (37°C) as infused during 15 min down the external carotid artery followed 10 min radioactive bolus mixture. The animals were decapitated 15 min later. Reversibility of the strapping was checked in 4 additional animals by administration of Evans blue 30 min after the 1 M sucrose, Evans blue as allowed to circulate for 20 min before the animal was sacrificed.

Disruption of the blood-brain barrier

4. 10 ml of blood (autotransfusion of blood slowly collected from the femoral artery inset) is rapidly injected (during 3) into the cannulated external carotid artery to reach the brain. The animals had been given Evans blue 20 min beforehand. The remaining 5 animals received no Evans blue and ethanol (see below) 10 min after the injection of blood and were

straction of urea was chosen from pilot study in which the concentration of urea was found to be that could open the structural blood-brain barrier as judged by macroscopic extravasation blue (9 of 10 animals, in 3 animals receiving 15 M urea no extravasation of Evans blue was observed; in 3 animals marked extravasation was obtained after 3 M urea).

study varying volumes of blood or Krebs-Ringer buffer solution were infused into different branches of the external carotid artery. A rapid injection during approximately 0.2 sec of 0.2 ml blood or non-oxygenated blood did not break the blood-brain barrier as judged by extravasation of Evans blue macroscopically detectable (4 of 5 animals). The same effect was achieved when 1 ml of blood or non-oxygenated blood was infused during 3 sec (5 of 5 animals). Injection of 0.04 ml Krebs-Ringer buffer solution or 1 ml of buffer solution during 15 sec did not induce any macroscopically detectable extravasation (4 of 4 animals). Consequently the rapid injection of 10 ml of blood was selected as the chance to disrupt the barrier mechanically.

killed 15 s later. In order to study reversibility of the mechanical barrier disruption, Evans blue injected 24 h after the autotransfusion of 1 ml blood during 3 s in 4 animals; in order to tracheotomy the cannulation of the external carotid artery was performed under bromal-sodium anaesthesia in these animals.

Fluorescence microscopy

The brain was removed from animals treated as above as well as from animals perfused with III through the left ventricle of the heart. Selected regions (frontal, parietal, temporal and occipital) or with underlying white matter caudate nucleus, thalamus, mesencephalon, and cerebellum) were rapidly in a propane-propylene mixture, cooled to the temperature of liquid nitrogen. After solvent freeze-drying, the specimens were processed for fluorescence microscopy according to the method of F. and Hillarp involving exposure of the tissue to gaseous formaldehyde during dry conditions (Falk and Özman 1972). The Evans blue-albumin complex displays a red fluorescence (Stern and Klatzo 1966) under the fluorescence microscopic conditions used.

Brain uptake index

Determination of the brain uptake index was performed by infusion during 1 s of 0.04 ml KI RI per buffer solution (37°C), containing a mixture of (-)-noradrenaline-7-³H (New England Nuclear specific activity 3.0 Ci/mmol concentration 7.5 nmol/ml) and ¹⁴C-ethanol (specific activity 51 mmol concentration 30 nmol/ml). The bolus mixture was infused through the cannula as centripetally in the right external carotid artery thus reaching the brain directly as described above. The heart of the animal was stopped with saturated potassium chloride 15 s after administration of the bolus, the animal was instantly decapitated and the head frozen in liquid nitrogen. Various brain regions (frontal, parietal and occipital cortex with underlying white matter caudate nucleus, thalamus, mesencephalon) from the injected side were dissected out in a cryostat at -15°C, weighed and put in liquid scintillation vials containing 0.5 ml Soluene (Packard). When the tissue had been dissolved, 1 ml of InstaGel was added. The tissue samples were counted for β-emissions in a Nuclear Chicago 1 scintillation counter. Quench correction was performed according to conventional principles.

To calculate the brain uptake index for noradrenaline the method developed by Oldendorf (1971) was used with two important modifications. Firstly the arterial inflow to the brain was not interfered with manipulation of the common carotid artery and secondly ¹⁴C-ethanol substituted for ³H₂O as internal standard ("freely" diffusible standard). The H and ¹⁴C ratio in the tissue is divided by the ratio in the injected mixture and the result multiplied by 100 to provide the uptake of the test substance as percentage of the ethanol uptake, thus

$$BUI = \frac{\text{tissue} - H / \text{tissue} - ^{14}C}{\text{mix} - H / \text{mix} - ^{14}C} \cdot 100$$

Cerebral blood flow

Determination of regional cerebral blood flow (rCBF) was performed by the ¹⁴C-ethanol technique according to Eklof *et al.* (1974). Catheters were placed into the femoral arteries and veins on both sides of the right external carotid was cannulated as described above (for further details, see Edvinsson 1977). 10 min after an injection of 2 M urea into the right internal carotid the rCBF was measured in 4 animals. The mean (±S.E.) flow in regions nourished by the carotid on the injected side was 66 ± 100 g/min, compared to 70 ± 8 on the contralateral side. Similarly rCBF was measured in 4 animals 10 min after a bolus injection of blood into the right internal carotid the mean flow was 72 ± 6 g/min on the injected side, compared to 74 ± 5 on the contralateral side. The slight reductions in flow on the injected side were not statistically significant.

Statistics

Differences between mean values were assessed by the Student's *t*-test for unpaired data.

Results

All brain regions taken from animals injected with Evans blue, but not treated to osmotic opening or mechanical disruption of the blood brain barrier showed no microscopically visible staining of the parenchyma. Fluorescence microscopy of these sections



Fig. 1. Sections of rat brain removed 10 min after injection of 2 M urea bolus into the right internal carotid artery. Patchy areas of extravasated Evans blue-albumin complex are seen in regions supplied by internal carotid artery.

sections revealed greenish background fluorescence in the brain tissue. The well-known pattern and distribution of the formaldehyde-induced fluorescence in the monoamine systems were recognized. The lumen of vessels everywhere in the brain contained Evans blue (in animals not perfused at sacrifice), which could easily be visualized by its bright red fluorescence. No Evans blue could be found in the vascular walls except for a fluorescence restricted to the intima of the pial vessels. After intracarotid injection of 2 M urea to induce osmotic opening of the barrier or an intracarotid injection of blood or buffer solution (1 ml during 3 s) to induce mechanical disruption of the barrier, a blue staining with patchy localization in individual animals was seen macroscopically in superficial and deep structures of the hemisphere on the injected side, whereas the brain stem including midbrain remained unstained (Fig. 1). These signs of extravasated Evans blue correspond to the regions reached by the initial high concentration of urea or by the initial high intracranial pressure induced by the rapid injection of blood or buffer solution. In the fluorescence microscope, a red fluorescence of patchy distribution was seen in all parts of the parenchymal regions which showed a macroscopically visible staining. In animals with mechanically induced disruption the leakage was noticed particularly at the level of small arterioles, capillaries and veins. The green formaldehyde-induced neuronal fluorescence in these regions of extravasation was not as well developed as in the remainder of the parenchyma; sometimes neurons were even seen to be loaded with red-fluorescent material (*cf.* Flodmark *et al.* 1974). Occasionally a red fluorescence was also seen in the wall of small vessels, particularly in the pial vessels, in the parenchyma outside areas showing extravasation. For obvious reasons, staining of the vessel wall was best seen in animals perfused at sacrifice. In animals with macroscopical extravasation of Evans blue, no sign of barrier damage—in the brain regions studied—appeared in the fluorescence microscope either. Reversibility of the damage was indicated by absence of extravasation of Evans blue (macroscopically and in the fluorescence microscope) in animals receiving the dye 30 min after 2 M urea, or 24 h after intracarotid injection of blood.

The brain uptake index for noradrenaline in animals with an intact blood-brain barrier varied according to the region studied, as shown in Table I. It was slightly higher in the superficial tissue than in the deeper regions of the hemisphere. After intracarotid injection of blood the brain uptake index for noradrenaline was markedly increased—by as much as 4 times—in those brain regions straightly reached by the essentially undiluted urea bolus (cerebral, occipital and frontal cortex preparations, caudate nucleus and thalamus). The brain uptake in these brain regions was enhanced from 3.6% to 13.8% (Fig. 2). Accordingly

killed 15 s later. In order to study reversibility of the mechanical barrier disruption, Evans blue was injected 4 h after the autotransfusion of 1 ml blood during 3 s in 4 animals; in order to tracheotomy the cannulation of the external carotid artery was performed under brief sodium anaesthesia in these animals.

Fluorescence microscopy

The brain was removed from animals treated as above as well as from animals perfused *in vivo* through the left ventricle of the heart. Selected regions (frontal, parietal, temporal and occipital cortex with underlying white matter, caudate nucleus, thalamus, mesencephalon, and cerebellum) were rapidly frozen in a propane-propylene mixture, cooled to the temperature of liquid nitrogen. After subsequent freeze-drying, the specimens were processed for fluorescence microscopy according to the method of Falck and Hillarp, involving exposure of the tissue to gaseous formaldehyde during dry conditions (Falck and Owman 1972). The Evans blue-albumin complex displays a red fluorescence (Stenroos and Klatzo 1966) under the fluorescence microscopic conditions used.

Brain uptake index

Determination of the brain uptake index was performed by infusion during 1 s of 0.04 ml/kg of Ringer buffer solution (37°C), containing a mixture of (-)-noradrenaline-7 H (New England Nuclear) specific activity 3.0 Ci/mmol (concentration 7.5 nmol/ml) and C-ethanol (specific activity 51 Ci/mmol) concentration 30 nmol/ml. The bolus mixture was infused through the external carotid artery centripetally in the right external carotid artery thus reaching the brain directly as described above. The heart of the animal was stopped with saturated potassium chloride 15 s after administration of the bolus, the animal was instantly decapitated and the head frozen in liquid nitrogen. Various regions (frontal, parietal and occipital cortex with underlying white matter, caudate nucleus, thalamus, and mesencephalon) from the injected side were chiselled out in a cryostat at -15°C, weighed and placed in liquid scintillation vials containing 0.5 ml Soluene (Packard). When the tissue had been dissolved, 10 ml of Instagel was added. The tissue samples were counted for β -emissions in a Nuclear Chicago LS 5000TD liquid scintillation counter. Quench correction was performed according to conventional principles.

To calculate the brain uptake index for noradrenaline the method developed by Oldendorf (1971) was used with two important modifications. Firstly the arterial inflow to the brain was not interrupted and manipulation of the common carotid artery and secondly ^{14}C -ethanol substituted for ^3H -ethanol as internal standard ("freely" diffusible standard). The ^3H and ^{14}C ratio in the tissue is divided by the ^3H and ^{14}C ratio in the injected mixture and the result multiplied by 100 to provide the uptake of the test substance as a percentage of the ethanol uptake, thus

$$\text{BUI} = \frac{\text{tissue} - \text{H/tissue} - ^{14}\text{C}}{\text{mix} - \text{H/mix} - \text{C}} \times 100$$

Cerebral blood flow

Determination of regional cerebral blood flow (rCBF) was performed by the ^{14}C -ethanol technique according to Ekblom *et al.* (1974). Catheters were placed into the femoral arteries and veins on both sides. The right external carotid was cannulated as described above (for further details, see Edvinsson *et al.* 1977). 10 min after an injection of 2 M urea into the right internal carotid the rCBF was measured in 4 animals. The mean (\pm S.E.) flow in regions nourished by the carotid on the injected side was 66 ± 10 ml/100 g min, compared to 70 ± 8 on the contralateral side. Similarly rCBF was measured in 4 animals 10 min after bolus injection of blood into the right internal carotid, the mean flow was 77 ± 6 ml/100 g min on the injected side, compared to 74 ± 5 on the contralateral side. The slight reductions in flow on the injected side were not statistically significant.

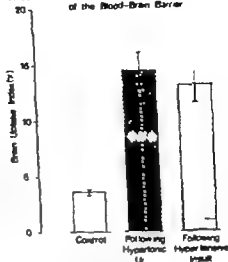
Statistics

Differences between mean values were assessed by the Student's *t*-test for unpaired data.

Results

All brain regions taken from animals injected with Evans blue, but not treated to osmotic opening or mechanical disruption of the blood brain barrier showed no macroscopically visible staining of the parenchyma. Fluorescence microscopy of these areas

Cerebral Uptake of Noradrenaline following Opening of the Blood-Brain Barrier



2. Mean values of brain uptake index for noradrenaline supplied by the internal carotid artery (Table I). Unrestricted controls and animals subjected to osmotic opening of the blood-brain barrier by 2M urea or mechanical disruption of the barrier by pulse of hydrostatic pressure. The difference between the mean values is highly significant ($p < 0.01$).

mechanically from the cerebral endothelial cells resulting in their shrinkage, which is held to transiently open up the barrier at the tight junctions between contiguous endothelial cells (Rapoport *et al.* 1972). The local concentration of urea must be high to have effect (Rapoport *et al.* 1972), in accordance with the present observation that no macroscopically or microscopically visible extravasation of Evans blue occurred in regions outside supply of the internal carotid artery (into which the urea bolus was injected), even though these regions were exposed to the urea diluted in the systemic circulation. The threshold concentration of the urea solution needed to induce barrier opening was in the magnitude of 2 M, which is in agreement with observations in the rabbit (Rapoport *et al.* 1972). It has been shown that the barrier opening was reversible within 30 min, confirming earlier findings by Rapoport *et al.* (1972).

A pulse of hydrostatic pressure, elevating the carotid artery pressure above 200 mmHg, has been shown to open the blood-brain barrier in the rat as evidenced by extravasation of Evans blue (Rapoport and Thompson 1975). This barrier damage was confirmed in the present series of expts. and the extravasation was predominantly noticed at the level of the pre-branch. Acutely induced extreme hypertension also causes blood-brain barrier leakage, preferentially in small arterioles and capillaries, probably related to pressure-induced overdistension of the vessels (Johansson, Strandgaard and Lassen 1974, MacKenzie *et al.* 1976 c). If it may be assumed that the same mechanisms is responsible for the mechanical barrier disruption induced by a pulse of hydrostatic pressure. At the ultrastructural level, barrier damage in acute hypertension comprises formation of channels in the cytoplasm of endothelial cells, an increased transendothelial pinocytosis and, rarely opening of the tight junctions (Hansson, Johansson and Blomstrand 1975). It is probable, but not yet shown, that the hydrostatic pressure-induced barrier damage has a similar ultrastructural appearance. Reversibility of barrier disruption was shown to occur within 24 h, the time interval used on the basis of similar expts. by Johansson (1974).

TABLE I Values for brain uptake of *H*-noradrenaline before and after osmotic opening or mechanical disruption of the blood-brain barrier by injection of 2 M urea or a rapid bolus of blood respectively into the internal carotid artery. Mean values \pm S.E. of 6 animals in each pre (for mesencephalon^b only 3 values were obtained).

Brain region	Intact barrier	After Osmotic opening	After mechanical disruption
Parietal cortex	4.26 \pm 0.44	16.61 \pm 3.00	15.61 \pm 3.46
Occipital cortex	4.46 \pm 0.45	17.53 \pm 3.59	14.09 \pm 2.93
Frontal cortex	3.40 \pm 0.29	11.40 \pm 1.97	9.78 \pm 1.66
Caudate nucleus	2.83 \pm 0.38	12.48 \pm 3.04	11.35 \pm .69
Thalamus	2.69 \pm 0.40	10.93 \pm 2.62	11.26 \pm 3.0.
Mesencephalon	3.98 \pm 0.46	3.93 \pm 0.49 n.s.	2.93 \pm 0.06 n.s.

Comparison of mean values according to Student's *t*-test 0.001 < *p* < 0.01 *0.01 < *p* 0.05 n.s. = non-significant.

^b That radioactivity sufficiently above back-ground reached mesencephalon at all, although this region is not directly supplied by the internal carotid artery may be explained by a transient change in the level of flow during injection.

the index was not affected in mesencephalon a region supplied by the vertebro-basilar system (Table I). Similarly after mechanical disruption of the barrier the brain uptake index was enhanced over 3-fold in those parts of the brain affected by the high luminal pressure induced by the rapid injection of blood (the mean uptake increased by 3.6% to 12.4% Fig. 2). As could be expected, the uptake was not enhanced in mesencephalon (Table I).

Discussion

The ability of many amines to penetrate from the circulation into the brain parenchyma is generally considered to be minimal, if any. Fluorescence microscopy after systemic administration of neurotransmitter amines, such as noradrenaline has indicated that the passage is predominantly impeded already at the luminal surface of all pial and parenchymal vessels but in addition at the level of endothelial cells and pericytes of cerebral capillaries and venules (Hardebo *et al.* 1977). There is evidence that monoamine oxidase, present in the vessel wall is involved in this barrier mechanism so that the monoamines are trapped by enzymatic breakdown process (Bertler *et al.* 1966, MacKenzie *et al.* 1976 b, Hardebo *et al.* 1977). There is no reason to believe that the monoamine barrier is directly associated with the particular ultrastructural organization of the cerebrovascular endothelium. Thus, for example, electron microscopy studies have not demonstrated any differences between endothelial cells and cellular interconnections on the arterial side on one hand and capillaries and veins on the other (Reese and Karnovsky 1967), although the passage of amine is different in these two parts of the vascular bed, as shown by fluorescence microscopy (Hardebo *et al.* 1977).

It has recently been shown that intravascular administration of hypertonic solutions, e.g. urea allows large molecules that do not normally penetrate the blood-brain barrier (like horseradish peroxidase and Evans blue-albumin complex) to pass from the blood to brain tissue (Rapoport, Hori and Klatzo 1972, Brightman *et al.* 1973). This was confirmed in the present study using Evans blue. It has been suggested that urea extracts intracellular

has been suggested (Edvinsson *et al.* 1972) that the low penetrability of noradrenaline across the intact blood-brain barrier may account for the relatively weak effect of the amine on central blood flow when injected into the circulation. This assumption has found direct support in experiments showing that osmotic opening of the barrier markedly alters the noradrenaline-induced effect on local cerebral blood flow (MacKenzie *et al.* 1976 a, Edvinsson *et al.* 1977).

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A useful method for quantitative measurement of the passage of a wide variety of solutes from blood to brain vessel walls and brain parenchyma has been developed by Oldendorf (1971). According to this method, the substance to be tested is injected in its radioactive form into the common carotid artery together with a radiolabelled highly diffusible compound used as internal standard, and the radioactivity is measured in the brain after a single capillary passage. Recently this method has been slightly altered by Oldendorf and Braun (1974). The original method was modified in the present study by employing ^{14}C -labelled ethanol instead of tritiated water as the internal standard, since it has been demonstrated that the latter diffuses significantly better into the brain (Raichle *et al.* 1976). Using tritiated water as standard, Oldendorf found a mean brain uptake index of 4.5 for noradrenaline, whereas in the present experiments, using ^{14}C -ethanol as standard, a value of 3.6 was obtained. This difference can be explained on the basis of the data given by Raichle *et al.* (1976), who found that only 93% of an injected bolus of labelled water is freely exchangeable with brain provided the cerebral blood flow is about 50 ml/100 g/min; the comparable figure for ethanol is 97%. At higher flow levels the extraction tends to be reduced. Catheterization of the external carotid artery on one hand and the injection of urea (Mackenzie *et al.* 1974) or a bolus of blood (see Methods) on the other did not cause any significant change in the cerebral blood flow at the point of time when the brain uptake index was estimated. Further, it is highly unlikely that the trace amounts of noradrenaline that are injected can themselves affect the CBF. This would mean that with the flows obtained in the present study, the absolute values for the brain uptake index will, if anything, be slightly underestimated. Since the use of ethanol as standard would, on the other hand, tend to slightly overestimate the true uptake of amines, the technique allows for a good approximation of the permeability of various test solutes. The brain uptake index for noradrenaline was found to vary between 2.7 and 4.5 depending on the region studied. A second modification of the Oldendorf technique comprised injection into the internal carotid artery via the cannulated external carotid and not through a needle in the common carotid artery. This procedure eliminates any interruption of blood flow through the internal carotid artery and also lowers the risk of vasospasm of this artery frequently occurring when the common carotid artery is manipulated.

The brain uptake index was utilized not only to determine the normal regional passage of noradrenaline into the brain, but also to quantify the impairment of barrier function induced by osmotic opening or mechanical disruption. It was found that in those regions of the brain reached by high concentrations of urea, as evidenced by extravasation of Evans blue (reflecting areas where the barrier had been disrupted mechanically), the brain uptake of noradrenaline was enhanced more than 3-fold. In both series of experiments, the relative increase in brain uptake was similar in all brain regions studied, indicating that the extent of barrier damage was of the same magnitude in these regions. Since the standard used, ethanol, is not freely permeable, as mentioned above, an opening of the barrier may tend to enhance the passage also of the standard and possibly influence the brain uptake index. However, any increase in the passage of ethanol can only be minimal, resulting in but a very slight underestimation of the index, and it may even be proportional to the increase in the passage of the test substance, and hence still give a correct estimation of the index.

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Low Intensity Training, Inactivity and Resumed Training In Sedentary Men

By

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Abstract

ÖLANDER, J. K.-H. KIEHLING, J. KARLSSON and B. EKBLOM. *Low-intensity training, inactivity and resumed training in sedentary men*. Acta physiol. scand. 1977 101 351-362.

Effects of low intensity training regimen, consisting of two 7-week periods with an interspersed rest/inactivity period were investigated in 16 sedentary men. A follow-up was made on 7 subjects after additional weeks' training. Systemic as well as local effects were studied using exercise tests and leg muscle biopsies. The two 7-week training periods both resulted in a 6% decrease in $\dot{V}O_{2\max}$ and lowered heart rates during submaximal work. No persisting training effects were detected by exercise tests after inactivity. However, striking differences in enzyme activity pattern and ultrastructure were observed between the two periods, indicating that some training effect of importance for muscle metabolism was maintained during inactivity. It is suggested that such an effect might be associated with the local oxygen supply. During the 38-week training period there was a large increase in muscle oxidative capacity but no change in maximal oxygen uptake. This separation of systemic and local training effects indicates lack of direct causal relationship between muscle metabolic potential and maximal $\dot{V}O_{2\max}$. It is suggested that the elevated muscle oxidative capacity is of importance for an increased aerobic capacity.

7-week Exercise, training, inactivity, oxygen uptake, muscle metabolism, muscle ultrastructure

It is well established that regularly performed endurance-type training will induce significant adaptations at the systemic as well as at the muscle cell level. Improvements in cardiovascular function (e.g. Sahlin *et al.* 1968, Ekbom 1969) are accompanied by an increased metabolic capacity in skeletal muscle, resulting in augmented working capacity and endurance (e.g. Åstrand and Booth 1976).

The circulatory effects of low intensity training have been evaluated in many studies—(Ekbom (1971) (for other references, see Pollock 1973). However, little attention has hitherto been paid to the effects on local muscle physiological factors of the kind of low-intensity training that can be carried out mainly unsupervised by previously sedentary people. The present investigation has therefore been concentrated on the following three points: (1) to study the effects of training of low intensity on sedentary people, (2) to establish

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By

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Effects of low intensity training regimes, consisting of two 7-week periods with an interspersed rest/inactivity period were investigated in 16 sedentary men. A follow-up was made on 7 subjects after 33-week rest/inactivity. Systemic as well as local effects are studied using exercise tests and leg muscle biopsies. The two 7-week training periods both resulted in a 6% increase in $\dot{V}O_2$ max and lowered heart rate during submaximal work. No persisting training effects were detected by exercise tests after inactivity. Skeletal muscle, however, striking differences in enzyme activity patterns and ultrastructure were observed between the two periods, indicating that some training effect of importance for muscle metabolism persists despite prolonged inactivity. It is suggested that such an effect might be associated with the local oxygen supply. During the 33-week training period there was a large increase in muscle oxidative capacity but no change in maximal oxygen uptake. This separation of systemic and local training effects indicates lack of direct causal relationship between muscle metabolic potential and maximal oxygen uptake. It is suggested that the elevated muscle oxidative capacity is of importance for an increased aerobic capacity.

Key words: Exercise, training, inactivity, oxygen uptake, muscle metabolism, muscle ultrastructure.

It is well established that regularly performed endurance-type training will induce significant adaptations in the systemic as well as at the muscle cell level. Improvements in cardiovascular function (e.g. Saltin *et al.* 1968, Ekblom 1969) are accompanied by an increased metabolic capacity in skeletal muscle, resulting in augmented working capacity and endurance (e.g. Saltin and Booth 1976).

The circulatory effects of low intensity training have been evaluated in many studies (e.g. Ekblom (1971) (for other references, see Pollock 1973)). However little attention has hitherto been paid to the effects on local muscle physiological factors of the kind of low intensity training that can be carried out mainly unsupervised by previously sedentary people. The present investigation has therefore been concentrated on the following three problems: (1) to study the effects of training of low intensity on sedentary people, (2) to establish

FK activity was estimated according to Shook and Borer (1964), LDH and HAD by the methods of *et al.* (1969), CK as described by Sorens (1969), and cytochrome according to Wherrett *et al.* (1969). m-GPD activity was estimated by two different methods: the first two biopsies were analyzed polarographically with Clark oxygen electrode and reaction mixture containing 30 mM potassium phosphate, pH 7.5, 10 mM cytochrome c, 4.5 mM m-glycophosphate, 1 μ M rotenone and 100 μ l homogenate in final volume of 1 ml. The following two biopsies were assayed according to Bass *et al.* (1969) as modified by Peter *et al.* (1970). m-GPD activity was not determined in the fifth biopsy. The spectrophotometrical assays are run in a Beckman model BS recording double beam spectrophotometer and polarographical assays made at 25°C.

ATPase was estimated according to Lowry *et al.* (1951). All reagents and enzymes used in the assays were purchased from Sigma Chemical Co or Boehringer Mannheim GmbH.

Microscopy. The muscle specimen was frozen in isopentane cooled by liquid nitrogen. Transverse sections (20 μ m) were cut in cryostat at -20°C, mounted on cover glasses, and stained for myofibrillar protein (Padykula and Herman 1955) after preincubation at pH 10.5 (for details, see Gollnick *et al.* 1972). Small pieces of muscle tissue were fixed in ice-cold 2% osmium tetroxide in veronal buffer, pH 7.4, for two hours, washed, dehydrated in ethanol and propylene oxide, and embedded in Epon. Thin sections, approximately 70-80 nm thick, were cut on LKB Ultratome I, stained with uranyl acetate and lead citrate and examined in Siemens Elmiskop 101 or Hitachi HU 11E1 electron micro-

graphs (final magnification about 24 000) were taken at random from 2-4 muscle fibres per section and analyzed stereologically according to Weibel (1969). To estimate the number of mitochondria per unit volume (N_V), the formula $N_V = K/\beta [N_A/V]$ (Weibel 1969) was applied. The size distribution of the mitochondria was taken to be 1.07 and the shape coefficient β to be 1.6 (each refers to spheroids with an area/volume ratio of 2). Mitochondrial mass volume was calculated by dividing the volume fraction V_V by the density of mitochondria. The technique gives only rough estimates of the true mitochondrial number and mass volume, since the actual muscle mitochondria often are irregularly shaped. For comparative purposes, however, the method is applicable.

Recently Essenberg and Kaden (1976) have shown that the three fibre types of guinea pig skeletal muscle can be identified in electron micrographs with 90 per cent success rate on the basis of Z-line width and mitochondrial density. Unfortunately we found neither bimodal Z-line width distribution nor relationship between Z-line width and mitochondrial volume when applying the same technique to 138 micrographs from our 16 subjects. This may be due to less clear-cut differences between the fibre types in human muscle. The line structure of the M-line is another parameter that has been used to discriminate between fibre types (Gjessing and Sorensen 1970). Such an analysis is prohibited by the insufficient resolution achieved in the electron micrographs of the biopsies. Therefore, no unambiguous identification of fibre types could be made.

It was, however, observed that, although the Z-line width was unimodally distributed within the total muscle, it was possible to discern two groups of Z-line widths in almost all individuals. By designating the fibres with broad Z-lines as slow twitch (ST) fibres (e.g. Wroblewski and Jansson 1975), the proportion of ST fibres was obtained for the whole subject group, that agreed within 2 per cent with the histochemically obtained slow fibre values. Thus, the morphometrical data can be regarded as representative for the whole muscle, even though the individual values may be affected by the low number of fibres analyzed.

Conventional statistical methods were applied. Student's *t*-test was used to test differences between values before and after training, $p < 0.05$ being considered significant. Correlations were shown by linear regression analysis.

Results

Exercise tests. The results from the exercise tests are shown in Table I. The group mean $V_{O_2 \max}$ was the same before the training started in period I and in period II. The group mean increase was also about the same in the two training periods, 5.1 and 5.9%, respectively (both $p < 0.05$). The HR at the submaximal work load was lower after each training period (both $p < 0.05$). The increase in oxygen pulse (V_{O_2}/HR) during the submaximal work was from 13.8 to 14.5 ml O_2 beat⁻¹ (5.1%, $p < 0.05$) in period I and from 14.5 to 15.2 ml O_2 beat⁻¹ (4.8%, $p < 0.05$) in period II.

TABLE I Body weight, oxygen uptake ($\dot{V}O_2$), heart rate (HR), blood lactate ([HLa]) and rate of perceived exertion (RPE) during submaximal and maximal exercise before and after each training or inactivity period. Note that the submaximal load was different during training periods I and II (see Materials and Methods). Values are means \pm S.E. * denotes significant difference ($p < 0.05$) when compared with the value from the respective training period.

Test occasion	n	Body weight kg	Submaximal exercise					Maximal exercise				
			$\dot{V}O_2$ l \times min	HR beats \times min	[HLa] mM	RPE, points		$\dot{V}O_2$ l min	HR beats min ⁻¹	[HLa] mM	RPE points	
						Local ^a	Central ^a					
Before training period I	16	77.0 ± 3.2	1.88 ± 0.0	136.9 ± 3.2	3.3 ± 0.3	11.8 ± 0.4	1.0 ± 0.3	3.37 ± 0.11	44 ± 1.2	193.4 ± 1.9	9.8 ± 0.5	
After training period I	16	77.3 ± 3.3	1.85 ± 0.02	137.2 ± 3.4	2.7 ± 0.3	11.2 ± 0.5	1.1 ± 0.5	3.40* ± 0.12	47.1 ± 1.3	189.6 ± 1.9	11.7 ± 0.6	
After inactivity period I	16	76.1 ± 3.0	1.44 ± 0.13	136.6 ± 4.8	7.0 ± 0.8	14.6 ± 0.6	13.3 ± 0.4	3.38 ± 0.1	44.7 ± 1.3	190.9 ± 1.9	10.8 ± 0.6	
After training period II	16	73.9 ± 2.9	1.44 ± 0.13	146.3 ± 4.4	5.7 ± 0.6	12.9 ± 0.7	12.0 ± 0.5	3.58 ± 0.14	47.5 ± 1.2	186.8 ± 2.6	11.3 ± 0.4	
Before training period III	7	80.2 ± 5.7	—	—	—	—	—	3.91 ± 0.13	49.9 ± 2.1	191.4 ± 3.1	11.3 ± 0.4	
After training period III	7	81.8 ± 4.9	—	—	—	—	—	3.84 ± 0.08	47.6 ± 2.7	191.3 ± 3.3	11.8 ± 0.7	

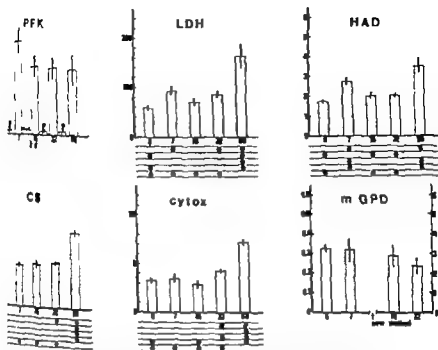
I and from 15.5 to 16.6 ml O_2 \cdot beat⁻¹ (7 l \cdot min⁻¹, $p < 0.05$) in period II. During both periods there was a tendency ($p < 0.05$) towards a reduced [HLa] and decreased "local" and central RPE scores at submaximal exercise. During maximal exercise HR, [HLa] and RPE were unchanged.

In the follow-up tests after period III there was a slight drop in $\dot{V}O_{2\max}$ in the 7 subjects from 3.91 l \cdot min⁻¹ to 3.84 l \cdot min⁻¹ ($p < 0.05$). Since the mean body weight increased somewhat during the period (from 80.2 to 81.8 kg) $\dot{V}O_{2\max}$ per kg body weight decreased from 49.9 to 47.6 ml \cdot min⁻¹ \cdot kg⁻¹ (6.2%, $p < 0.05$). HR, [HLa] and RPE scores at maximal exercise were unchanged.

[Hb] and Hct values at rest and during exercise were not affected by the training regimen.

Fibre composition. The percentage of slow twitch oxidative fibres (%ST) in the rectus abdominis was unaffected by the training regimen, viz. 39.7 \pm 3.7 (15), 35.8 \pm 4.0 (12), 36.0 (14), 39.9 \pm 2.2 (15) and 39.4 \pm 5.2 (7) (means \pm S.E., number of subjects within brackets) before and after period I, after inactivity, after period II and after period III respectively. Some biopsies could not be analyzed for technical reasons. No attempts to subdivide slow twitch fibres into A, B and C groups (Brooke and Kaiser 1970) were made.

The data allowed an assessment of the degree of variability in fibre composition between biopsy specimens taken at different times and sites from the same muscle. The mean difference between biopsies was 7.5 \pm 6.0 (S.D.) %ST (48 pairs) which was almost identical



Enzyme activities in skeletal muscle before and after each training or inactivity period. Columns represent S.E. for 16 subjects (7 at 40 weeks). The activities are given as $\mu\text{mol min}^{-1} (\text{g wet wt})^{-1}$ for phosphofructokinase (PFK), lactate dehydrogenase (LDH), 3-hydroxyacyl-CoA dehydrogenase (HAD), citrate synthase (CS), and as $\mu\text{mol O}_2 \text{ min}^{-1} (\text{g wet wt})^{-1}$ for cytochrome oxidase (cyto). For mitochondrial glycerophosphate dehydrogenase (m-GPD), the first two values are given as $\mu\text{mol O}_2 \text{ min}^{-1} (\text{g wet wt})^{-1}$ and the last two as $\mu\text{mol min}^{-1} (\text{g at } 1) \text{ min}^{-1}$ (for details, see Materials and Methods section). Small differences ($p < 0.05$) are indicated by asterisks below each diagram.

the corresponding value when biopsies taken from alternate legs in the same subject compared (7.4 ± 6.0 %ST, 73 pairs). This variability was slightly greater than the one noted by Gollnick *et al.* (1972), a fact possibly due to differences in the size of the analyzed samples. Since the mean %ST was 38.0 in each of the legs (all biopsies included), the data indicate that the two vastus lateralis muscles in the same individual may be considered as identical as far as fibre composition is concerned.

During period I there was a correlation between the %ST and the increase in $\dot{V}_{O_2 \text{ max}}$ ($p < 0.05$). No such correlation was present during periods II and III or when the two periods were treated together.

Energy metabolism. The effects of the various periods of training and inactivity on the rates of the 6 selected enzymes are shown in Fig. 1.

The first training period resulted in significant increases in the activities of LDH and HAD, reflecting anaerobic capacity and fatty acid β -oxidation, respectively. HAD dropped slightly during the inactivity period, and was then unaffected by the second training period. During the latter period mitochondrial oxygen consumption capacity represented by $\dot{V}_{O_2 \text{ max}}$ increased by 50 per cent. Furthermore, the activities of LDH and of the citric acid cycle enzyme CS were elevated as compared to before the first training period. The 38 sub-

sequent weeks of self-governed training (period III) produced large increases in LDH, HAD, CS and cytox activities, indicating further augmentations both in anaerobic capacity and the capacity to oxidize fatty acids. The glycolytic pathway represented by PFK, was not affected by the training regimen. m-GPD representing the glycerophosphate shuttle, showed no effects of training.

Pette and coworkers (Bass *et al* 1969, Staudte and Pette 1971) have demonstrated a pattern of functional coordination. One of the most prominent correlations is the one between CS and HAD. In the present material, this correlation was found before ($r = 0.81$, $p < 0.01$) and after ($r = 0.81$, $p < 0.001$) the first training period, and at the 60 week time occasion ($r = 0.90$, $p < 0.001$). No significant correlation was, however, present before ($r = 0.33$) or after ($r = 0.39$) the second training period, suggesting a loss of coordination.

Studies on mixed human muscles with differing fibre composition, and on isolated muscle fibres (Essén *et al* 1975, Sjödin 1976, Thorstensson 1976) have shown that slow twitch and fast twitch fibres differ in their metabolic profiles. The present data failed to reveal any relationships between fibre composition and enzyme activities, with the exception of LDH versus %ST after period I ($r = -0.62$, $p < 0.05$) and m-GPD versus %ST before period II ($r = -0.70$, $p < 0.01$).

The muscle protein content changed in response to training and inactivity and was 11.4 \pm 0.5 (16), 13.4 \pm 0.9 (16), 11.6 \pm 0.6 (16), 14.1 \pm 0.7 (16) and 9.3 \pm 0.6 (7) (per cent of wet weight, means \pm S.E., number of subjects within brackets) at the start of the trial, at period I, after inactivity after period II and after period III respectively. The protein content was significantly higher after period II than before periods I and II. The last (week) value was significantly lower than all the other values. This was not due to a low protein content in the 7 subjects before period III (mean value 13.7%).

Muscle ultrastructure. Data on volume fraction, number per unit volume of sarcomere and mean volume of mitochondria, as well as the volume fraction of lipid droplets before and after each training or inactivity period are given in Fig. 2.

The only observed ultrastructural effect of the first training period was a doubling of intracellular triglyceride content. This was, however, restored to pretraining levels after the inactivity period, and no corresponding increase was observed during the subsequent training periods. During the second training period, the mitochondrial density in the fibrillar space tended to increase ($p < 0.1$) and at 60 weeks it was significantly greater than before training. This increase seemed to be due mainly to a rise in mitochondrial number, indicated by a tendency towards an increased mitochondria-to-sarcomeres ratio (N_m , Fig. 2, lower left).

Essentially, the data showed no clear-cut effects of the training regimen on subsarcolemmal mitochondria. This may be a true phenomenon, but no definite conclusions can be drawn because of the relatively sparse occurrence of subsarcolemmal zones in the micrographs, resulting in a small sample.

A correlation between mitochondrial density in the fibrillar space and cytox activity was present after the first ($r = 0.53$, $p < 0.05$) and second ($r = 0.64$, $p < 0.05$) training periods.

Smokers vs. non-smokers. Before training period I, smokers ($n = 8$) had significantly lower cytox activities (2.60 ± 0.26 vs. 4.02 ± 0.32 $\mu\text{mol O}_2 \text{ min}^{-1} \times \text{wt}^{-1}$, means \pm S.E.).

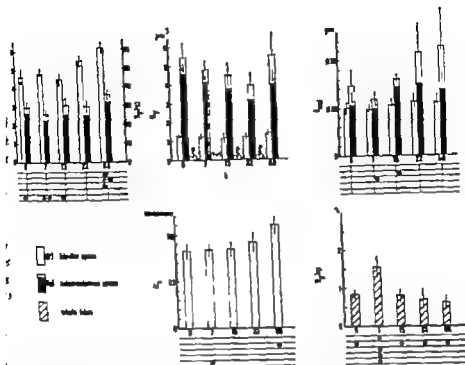


Fig. 2. Volume fraction (V), number per unit volume (N_p) and mean volume (V_{mb}) of mitochondria in fibrillar and subsarcolemmal spaces of skeletal muscle fibres, number of mitochondria per sarcomere (N_p) and volume fraction of lipid droplets (V_{lipid}) before and after each training or inactivity period. Columns show means ± 3 S.E. for 16 (8 weeks), 15 (7 weeks), 10 (15 weeks), 10 (22 weeks) and 7 (60 weeks) subjects. Significant differences ($p < 0.05$) are indicated by asterisks below each diagram. Note that numbers and mean volumes of mitochondria are estimates of the true values (see Materials and Methods section).

and fibrillar space mitochondrial densities (3.90 ± 0.34 vs. 5.45 ± 0.47 , means \pm S.E.) than non-smokers ($n=8$). For cytosol, this difference persisted during period I, and then disappeared. No other significant differences between smokers and non-smokers in any studied parameter were observed at any time during the investigation.

Discussion

Oxygen transport system. In the present investigation, the "classical" effects of a physical training programme on circulation were demonstrated. Thus, in parallel with the improved physical performance capacity as judged from work time and work load during the maximal exercise tests, the maximal oxygen uptake increased and the heart rate and rate of perceived exertion during the submaximal work load decreased after as compared to before each training period in all subjects. Furthermore, during the physical inactivity between the first and second training periods these values returned to pre-training levels.

The increase in $V_{O_{2\max}}$ was almost identical during the two training periods (Table I). However, there was no intraindividual correlation between the increases in maximal aerobic

sequent weeks of self-governed training (period III) produced large increases in LDH, HAD, CS and cyto x activities, indicating further augmentations both in anaerobic capacity and in the capacity to oxidize fatty acids. The glycolytic pathway represented by PFK, was unaffected by the training regimen m-GPD representing the glycerophosphate shuttle, showed no effects of training.

Pette and coworkers (Bass *et al* 1969, Staudie and Pette 1971) have demonstrated correlations between the activities of enzymes from different metabolic pathways, suggesting a pattern of functional coordination. One of the most prominent correlations is the one between CS and HAD. In the present material, this correlation was found before ($r=0.73$, $p<0.01$) and after ($r=0.81$, $p<0.001$) the first training period, and at the 60 week biopsy occasion ($r=0.90$, $p<0.001$). No significant correlation was, however, present before ($r=0.33$) or after ($r=0.39$) the second training period, suggesting a loss of coordination.

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The muscle protein content changed in response to training and inactivity and was 11.4, 10.5 (16), 13.4 ± 0.9 (16), 11.6 ± 0.6 (16), 14.1 ± 0.7 (16) and 9.3 ± 0.6 (7) (per cent of wet weight means \pm S.E., number of subjects within brackets) at the start of the trial, after period I, after inactivity, after period II and after period III respectively. The protein content was significantly higher after period II than before periods I and II. The last (60 week) value was significantly lower than all the other values. This was not due to a low protein content in the 7 subjects before period III (mean value 13.7%).

Muscle ultrastructure. Data on volume fraction, number per unit volume or sarcomere and mean volume of mitochondria as well as the volume fraction of lipid droplets before and after each training or inactivity period are given in Fig. 2.

The only observed ultrastructural effect of the first training period was a doubling of the intracellular triglyceride content. This was, however, restored to pretraining levels after the inactivity period, and no corresponding increase was observed during the subsequent training periods. During the second training period, the mitochondrial density in the fibrillar space tended to increase ($p<0.1$) and at 60 weeks it was significantly greater than before training. This increase seemed to be due mainly to a rise in mitochondrial number, indicated by a tendency towards an increased mitochondria-to-sarcomeres ratio (N_s , Fig. 2, lower left).

Essentially the data showed no clear-cut effects of the training regimen on subsarcolemmal mitochondria. This may be a true phenomenon, but no definite conclusions can be drawn because of the relatively sparse occurrence of subsarcolemma zones in the micrographs resulting in a small sample.

A correlation between mitochondrial density in the fibrillar space and cyto x activity was present after the first ($r=0.53$, $p<0.05$) and second ($r=0.64$, $p<0.05$) training periods.

Smokers vs. non-smokers. Before training period I smokers ($n=8$) had significantly lower cyto x activities (2.60 ± 0.26 vs. 4.07 ± 0.32 $\mu\text{mol O}_2 \text{ min}^{-1} (\text{g wet wt})^{-1}$ means \pm S.E.

—plasma, parallel variations in intracellular triglyceride content (Fig. 2) might, however, be a clue to a possible explanation. These effects might be indicative, not of a large training-induced increase in the capacity to utilize fatty acids, but of a substrate induction phenomenon, due to excessive mobilization of fatty acids from adipose tissue in response to the unusual exercise stress. This view is supported by the fact that a high-fat diet causes similar rises in human skeletal muscle (Esmén and Jansson, personal communication). Such a response may be transient, thus explaining the lack of a similar response during the old training period. The lack of an increased activity of HAD as during period II does not exclude an augmented fatty acid oxidation capacity (Bylund *et al.* to be published), since other factors than the β -oxidation enzymes, such as membrane passages, may rate-limiting.

In most studies in the literature, the activity of LDH is not influenced by endurance-type work (for review see Gollnick and Hermansen 1973). In the present study increases were observed after every training period, suggesting a elevated anaerobic capacity (Fig. 1). This discrepancy may be due to at least two factors. Firstly the pre-training LDH activities were generally low. The same observation has been made by Bass *et al.* (1976), who found significantly lower LDH activities in the *vastus lateralis* of sedentary men than in sporting or athletic groups. Secondly the training programme contained exercises that presumably tested the anaerobic work capacity namely callisthenics and, in the subjects who were released after period III, strength training. Strength-trained athletes tend to have higher LDH activities in their muscles than untrained subjects (Karlsson *et al.* 1975).

Cyt c activity was unaffected by the first seven weeks of training, but rose significantly during the other training periods (Fig. 1). This course of events was paralleled by the mitochondrial density in the fibrillar space (Fig. 2), indicating a close mitochondrial structure-enzyme relationship. These observations are in accordance with a previous study (Kjessling *et al.* 1974), where the cytochrome oxidase activity increased only 9% during eight weeks of moderate training, whereas seven additional weeks produced a further 65% increase. These results suggest the necessity of a rather long period of moderate training to produce changes in mitochondrial oxygen consumption capacity. During the "lag period" extra-synaptic changes in favour of an increased aerobic metabolism may occur. These changes may include improved blood supply by circulatory adjustments and increased capillarization (Andersen 1975), and augmented intracellular oxygen supply by a rise in myoglobin content (Pattengale and Hollway 1967). Persistence of such changes over the inactivity period would provide an explanation for the difference between the first and second training periods.

Improved aerobic conditions during prolonged muscular work may be an essential factor for the induction of aerobic enzymes like cytochrome oxidase and for the increase in mitochondrial density. Aerobic conditions have been shown to favour the production of the heart specific LDH isoenzyme (Dawson *et al.* 1964) and the synthesis of inner mitochondrial membrane components in heart and liver of newborn rats (Hallman 1971). Furthermore, it is conceivable that slight hypoxia during muscular work due to chronic carbon monoxide poisoning, may explain the fact that the smokers in this study had lower cyto c activities and mitochondrial densities than non-smokers before the training started. The disappearance of

power during training periods I and II ($r = 0.23$). The reason for this could be the relatively low training intensity and the small amount of training carried out in this study. The increase in maximal aerobic power was only about 6% in each training period, which is about the same as in the corresponding study of Kilbom (1971) but less than expected from other training studies with corresponding length of training period and age range but higher training intensity (e.g. Hartley *et al.* 1969). Since the training intensity rarely taxed the oxygen transport system above 80% of maximum and about one third of the training sessions were aiming to increase muscle power rather than maximal aerobic power variations in individual training intensity and attendance as well as other factors may play a more important role in this investigation as compared to other studies (e.g. Hartley *et al.* 1969).

It is also interesting to note that although it has previously been shown that individuals with the lowest pretraining values attain the greatest increase in $\dot{V}_{O_2 \max}$ during a short-term training programme (summarized in Saltin *et al.* 1969), the increase in $\dot{V}_{O_2 \max}$ in the present study was not correlated to the pretraining value. This may again be explained by the fact that the amount of physical training carried out was fairly small, not utilizing the whole increase potential in maximal aerobic power present in untrained individuals. In this respect there were no differences between individuals, who never had been physically active and those, who more than 10 years ago had been engaged in regular physical training.

Seven weeks of low intensity training was evidently not enough to prevent the oxygen transport system from returning to pre-training values during 8 weeks of inactivity. The seven weeks of renewed physical training increased maximal aerobic power to the same extent as did the first training period for the group as a whole, however with a large individual variation (cf. above). Thus, the previous training period did not influence the effects of the second training period on maximal aerobic power.

Muscle metabolism and ultrastructure The low-intensity training regimen employed was clearly sufficient to elicit significant metabolic adaptations in the *m. vastus lateralis*. The pattern of adaptation differed considerably between the three periods of training—a fact that appears to be due to (a) the duration of the training period and (b) effects of the previous training period.

Firstly the much more pronounced changes over a long period of low intensity training as opposed to a short one are obvious when comparing the different training periods (Fig. 1 and 2). Adaptation to prolonged low intensity training thus seems to be a gradual, long term process as far as skeletal muscle metabolic capacity is concerned. There were, however, some interesting short-term effects that will be discussed later on.

Secondly some of the differences in training response between the identical first and second training periods may possibly be due to persisting effects over the inactivity period.

Several investigations have demonstrated an increased fatty acid oxidation capacity after training (e.g. Baldwin *et al.* 1972, Molé and Holloszy 1970, Molé *et al.* 1971). In the present study increases in the activity of the β -oxidation enzyme HAD were observed twice (Fig. 1). The rise during the last, prolonged training period is in concert with the elevated overall oxidative capacity and probably reflects a significant increase in fatty acid combustion capacity. The significance of the 58% increase during the first training period is somewhat more obscure, since it did not reoccur when training was resumed after inactivity. The

out, parallel variations in intracellular triglyceride content (Fig. 2) might, however, be a possible explanation. These effects might be indicative, not of a large training-induced increase in the capacity to utilize fatty acids, but of a substrate induction phenomenon to enhance mobilization of fatty acids from adipose tissue in response to the unaccustomed stress. This view is supported by the fact that a high-fat diet causes similar increases in human skeletal muscle (Eaton and Jansson, personal communication). Such a response may be transient, thus explaining the lack of a similar response during the training period. The lack of an increased activity of HAD during period II, does not exclude an augmented fatty acid oxidation capacity (Bylund *et al.* to be published) other factors than the β -oxidation enzymes, such as membrane passages, may be limiting.

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LDH activity was unaffected by the first seven weeks of training, but rose significantly after other training periods (Fig. 1). This course of events was paralleled by the mitochondrial density in the intermyofibrillar space (Fig. 2), indicating a close mitochondrial structure-enzyme relationship. These observations are in accordance with a previous study (Kjessling 1974), where the cytochrome oxidase activity increased only 9% during eight weeks of endurance training, whereas seven additional weeks produced a further 65% increase. Both suggest the necessity of a rather long period of moderate training to produce a significant increase in mitochondrial oxygen consumption capacity. During the "lag period" extra changes in favour of increased aerobic metabolism may occur. These changes include improved blood supply by circulatory adjustments and increased capillarization (Patek and Holloszy 1975), and augmented intracellular oxygen supply by a rise in myoglobin (Patek and Holloszy 1967). Persistence of such changes over the inactivity period would provide an explanation for the difference between the first and second training periods.

Unaccustomed aerobic conditions during prolonged muscular work may be an essential factor for the induction of aerobic enzymes like cytochrome oxidase and for the increase in mitochondrial density. Aerobic conditions have been shown to favour the production of the specific LDH subunit (Dawson *et al.* 1964) and the synthesis of inner mitochondrial membrane components in heart and liver of newborn rats (Hallman 1971). Furthermore, it is probable that slight hypoxia during muscular work, due to chronic carbon monoxide poisoning, may explain the fact that the smokers in this study had lower cytochrome activities and mitochondrial densities than non-smokers before the training started. The disappearance of

these differences during the training also points to improved aerobic conditions as being a result of the training.

An increased muscle protein concentration in response to training was observed after the second training period. This has previously been seen in humans (Bylund *et al.*, to be published) but not in rats (Askew *et al.* 1973, Winder *et al.* 1974). In contrast to this, the protein content was lowered after the prolonged training period, which partly consisted of strength type training. Hypertrophy due to chronic overload has been noted to cause a decrease in protein concentration in rat skeletal muscle (Iannuzzo and Chen 1976).

Slow and fast twitch human skeletal muscle fibres differ in metabolic profile, as revealed by single fibre analyses (Essén *et al.* 1975, Sjödin 1976, Thorstensson 1976). These fundamental differences may give rise to significant correlations between the percentage of a fibre type and e.g. enzyme activities in mixed muscle samples with a range of fibre composition (e.g. Taylor *et al.* 1974, Thorstensson 1976). In the present material two such correlations were found (LDH *vs.* %ST after the first and m-GPD *vs.* %ST before the second training period). Since most metabolic parameters are susceptible to environmental influences such as training, individual variations in these respects probably obscure the fibre type dependence in most cases.

Systemic vs. local adaptation The results of the present investigation apparently suggest a separation of systemic and local training effects. The data from training periods I and II show the same improvement in oxygen uptake whether there is an increased skeletal muscle oxidative capacity or not. The separation is even more obvious during period III, with a large increase in muscle metabolic capacity but no change in maximal aerobic power. It thus appears possible, by low intensity training, to increase the oxygen uptake capacity to some extent without a simultaneous change in the oxidative potential of the working muscles. Conversely it appears possible to increase muscle metabolic capacity without a change in maximal oxygen uptake. In view of these observations, no direct causal relationship seems to exist between maximal oxygen uptake and muscle metabolic capacity in sedentary or moderately trained men.

It does not seem undue to speculate that the primary factor limiting the maximal oxygen uptake during running in sedentary individuals might be associated with the supply of oxygen to the working muscle cells. During training, improved oxygen delivery together with the increased energy demands then may induce an elevated muscle metabolic capacity which in turn calls for an improved function of the oxygen transport system, and so forth.

Since the increased muscle oxidative potential observed after training periods II and III was not associated with an increase in maximal aerobic power its significance must be sought elsewhere. Most probably this adaptive response is related to an improved endurance, enabling the muscles to work longer at a submaximal level by shifting the energy metabolism towards a greater fatty acid utilization, thereby sparing muscle and liver glycogen stores.

As already mentioned, the low intensity training used in this study brought about only a fraction of the possible increase in maximal aerobic power. Apart from training intensity, attendance and related factors, the increase potential is undoubtedly dependent upon the individual genetic endowment. Consequently one might expect those with the highest proportion of oxidative (ST) muscle fibres to attain the greatest increase in oxygen uptake.

training programme. This was actually the case during the first training period, but not II or III or the various periods together were considered, no such relationship established. Thus, during low intensity training, factors other than the geometrical ones are obviously decisive for the actual change in aerobic power. With an increasing intensity a more prominent correlation between the muscle fibre composition and the increase in maximal oxygen uptake might be expected.

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Structural "Resetting" of the Coronary Vascular Bed in Spontaneously Hypertensive Rats

By

E. NORESSON, M. HALLBÄCK and Å. HJALMARSSON

size and functional importance of the "structural autoregulation" which characterizes precapillary resistance vessels in hypertension, has been explored and documented in man and in rats (*cf* Folkow *et al.* 1973, 1974). Basically it constitutes a local response to sustained rises in average tissue load, and in the resistance vessels it takes the form of rapidly established media thickening once the average arterial pressure starts to rise. In the coronary bed, hypertrophy is associated with narrowing of the inner radius, so that the net increase in wall radius ensues. The same principle of tissue adaptation affects systemic arteries and the left heart. Whereas the rebuild of the resistance vessels leads to an increased flow resistance, that of the systemic arteries contributes to baroreflex resetting (*cf* Jones 1977) and the left ventricular hypertrophy to a functionally shifted Frank-Starling relationship (Hallbäck, Isaksson and Norrison 1975). During the various systemic circuits, the hemodynamic consequences of this structural adaptation have so far been analysed mainly in the vascular beds of skeletal muscle, gastrointestinal tract and kidneys, using spontaneously hypertensive rats (SHR) as a model of chronic hypertension (*cf* Folkow, Hallbäck and Norrison 1977, Folkow *et al.* 1977). Detailed analysis has, however, been performed on the coronary vascular bed where the adaptation is especially complex since the myocardium simultaneously undergoes a trophic adaptation. The present study is an attempt to explore in which way the coronary resistance is altered concerning dimensions and reactivity in the course of hypertension by comparing some hemodynamic characteristics of the coronary vascular beds in isolated hearts from SHR and normotensive control rats (NCR).

and 15 pre-scratched NCR rats body weight around 350 g were used. The SHR were in the phase of sustained hypertension and mean arterial pressure during waking conditions was 158 ± 6 mmHg in SHR and 105 ± 5 mmHg in NCR.

The structure of the coronary vascular bed was performed in a system for retrograde perfusion of the coronary bed (*cf* Folkow *et al.* 1967) consisting of an oxygenating chamber and two parallel-coupled perfusion ports. Three constant-flow pumps perfused the heart and the other one was used to allow the perfusion of Krebs bicarbonate buffer (100 ml) to be fully oxygenated in the chamber. Under anaesthesia the rat hearts were excised and immediately put into ice-chilled saline. Within one minute a steel cannula was inserted in the aorta towards the heart and the blood was thoroughly washed out before the cooled hearts were connected to the recirculating system for coronary perfusion. Flow was measured at the level of the aortic root by a transducer recording system. Flow could be set at

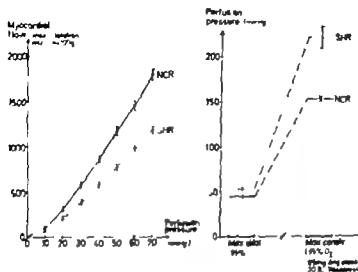


Fig. 1. Compiled results of 14 SHR and 15 NCR. Vertical line indicate SL. Left panel shows the pressure-flow relationship of the coronary vascular beds of SHR and NCR. At all pressure levels indicated flow is significantly lower ($p < 0.05$) in SHR than in NCR, except at the pressure of 10 mmHg. Right panel shows the pressure levels at maximal dilatation and maximal constriction is a constant flow of 13.2 ml/min.

any wished level) and the resistance of the coronary vascular bed could hence be calculated at different levels of pressure and flow.

Initially the perfusion medium was well oxygenated, i.e. gassed with 5% CO_2 in oxygen and flow was kept at 13.2 ml/g/min. When a steady pressure level was obtained, supramaximal amounts of vasopressin (20 IU) and of angiotensin II (0.5 mg), were added to the 100 ml perfusate to estimate the maximal pressor response, i.e. the maximal contractile strength of the coronary resistance vessels.

After changing the perfusate so that the pressor agents were eliminated, the new perfusate was again gassed with 5% CO_2 in nitrogen. The coronary vessels were then considered maximally dilated, but we checked by adding adenosine (50 μM). Flow was altered in a random fashion so that the pressure-flow relationship for the completely relaxed coronary resistance vessels could be determined. For each level in curve, relating pressure to flow/g tissue/min, was deduced.

During anoxic perfusion and maximal coronary vasodilatation, pressure-flow curves were constructed to characterize the dimensional design of the coronary vascular beds in SHR and NCR and the mean values are presented in Fig. 1 (left panel). As seen from the Figure the SHR curve is shifted to the right, and flow increases relatively less for a given pressure increase than the NCR one. Thus, at any given distending pressure the SHR resistance vessels exhibit a higher resistance per g of myocardium than the NCR ones also at complete relaxation. This indicates a structurally determined reduction in internal radius of the SHR coronary vessels. Further the SHR coronary resistance vessels are less distensible than the NCR ones which indicates thicker vascular walls in SHR (cf. Hallbäck, Lundgren and Åstrand 1974).

When the hearts were perfused at normal oxygen tension at a constant flow level addition of supramaximal doses of vasopressin and angiotensin caused a significantly greater increase in arterial perfusion pressure in SHR, 169 ± 11 mmHg, than in NCR, 105 ± 13 mmHg ($p < 0.001$) (Fig. 1 right panel). Thus the maximal levels of arterial perfusion pressure reached in SHR was 221 ± 12 mmHg and in NCR 153 ± 3 mmHg, a 45 per cent difference. The ability to produce a greater maximal contractile force suggests the presence of a correspondingly greater bulk of contractile media tissue in the SHR coronary resistance vessels than in the NCR ones, as discussed elsewhere (cf. Folkow *et al.* 1973). Evidently the coronary circuit in SHR exhibits the same type of adaptive structural change as in other systemic circuits.

of the myocardium this has, among other things, the consequence that at the same pressure the maximal coronary flow capacity per unit tissue weight is lower in hypertensive than in normotensive subjects. This may have serious consequences, particularly if too rapid reduction in mean arterial pressure is induced in hypertensive subjects by vasodilator therapy. It also raises the question whether this structural autoregulation of the coronary bed can be forced to regress towards normal in the course of a more sustained and gradual reduction caused by antihypertensive therapy.

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Vasoconstrictor Effect of Thromboxane A₂

By

JAN SVENSSON and BERTIL B. FREDHOLM

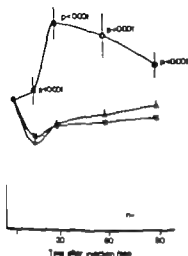
An unstable intermediate in the metabolism of the prostaglandin endoperoxides PGH₂ and PGG₂ was recently discovered (Hamberg *et al* 1975). There is considerable evidence that this compound, termed thromboxane A₂ (TxA₂), plays a role in the platelet release reaction and in platelet aggregation (Hamberg *et al* 1975, Svensson *et al* 1976). It has been shown that this material constitutes the major part of the rabbit aorta contracting substance detected by Piper and Vane (1969) following anaphylaxis in the guinea pig lung (Hamberg *et al* 1975) and is different from the prostaglandin endoperoxides (Svensson *et al* 1976). When human platelets are caused to aggregate by arachidonic acid or thrombin a substance which causes constriction of coronary artery strips is released. This material appears to be TxA₂ (Svensson and Hamberg 1976, Ellis *et al* 1976). TxA₂ is some 10-100 times more potent than its parent compound PGH₂ in causing constriction of rabbit aorta and some coronary arteries (Needleman *et al* 1976, Svensson and Hamberg 1976). A role for TxA₂ as a vasoconstrictor following trauma has therefore been proposed (Needleman *et al* 1976, Ellis *et al* 1976, Svensson and Hamberg 1976). In the present study we have attempted to study the vasoconstrictor effects of TxA₂ under *in vivo* rather than *in vitro* conditions.

The experiments were performed on 5 cats (2.8-3.7 kg) and 3 dogs (10-13 kg) anesthetized with pentobarbital. Blood pressure and heart rate were recorded by means of a Statham P23AC pressure transducer and a Grass polygraph. Subcutaneous adipose tissue (Rosell 1966) and gracilis muscle blood flows were recorded in dogs by means of arterial drop counters. Mesenteric and femoral blood flows were determined in cats.

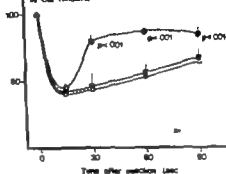
TxA₂ was generated, as described previously (Svensson *et al* 1976), by incubating human platelets (10⁹/ml) with arachidonic acid (5 µg/ml) for 30 min at 37°C. The mixture was rapidly filtered through a Millipore filter (0.45 µm). The filtrate was injected close to the aorta in a volume of 0.3-0.5 ml. For comparison PGE₂, PGH₂, arachidonic acid, ADP and frozen and thawed platelets were similarly injected. Statistical comparisons were performed using the Wilcoxon U-test.

The main findings of the present study are summarized in Fig. 1. It is evident that platelet filtrates that have been standing for 90 s or more produce profound vasodilatation in the vascular beds studied. This relaxation could be mimicked by frozen and thawed platelets and may therefore be due to some natural constituent of platelets. One such constituent, ADP, produced a similar vasodilatation in amounts of 45-100 nmol. Platelets release about 2

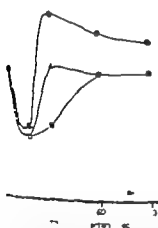
1 Cat mesentery



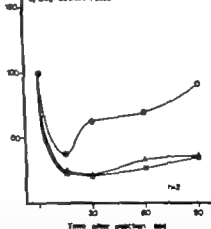
b/ Cat hindlimb



1 Dog gracilis



b/ Dog adipose tissue



1 Effect of platelet filtrate on the vascular resistance in a) cat mesentery b) cat hindlimb, c) dog gracilis and d) dog adipose tissue. A washed platelet suspension was incubated with arachidonic acid as described in methods. After filtration the platelet filtrate was incubated for 0-20 (O—O), 90-150 (—Δ) and 0-20 (—■). The vascular resistance 60 min after injection of the first (Tx_{A₂}-containing) as 96% 1 min after the latter (without Tx_{A₂}) in cat mesentery, 60 min higher in the cat hindlimb, 97 min higher in dog gracilis muscle and 170 min higher in the dog subcutaneous adipose tissue.

1 ADP 10⁻⁶ cells during aggregation (S. Ensson *et al.* 1976) i.e. the number of platelets in 1 ml incubate. 3 contrast PGE₂ and PGD₂ had to be given in amounts of 1 μg or 10 μg to produce a similar vasodilatation, and arachidonic acid itself produced negligible effects even in amounts four times as large as those added to the platelet suspension. 4 H (1 μg i.a.) produced a clearcut vasodilatation in dog gracilis and adipose tissue and in

Vasoconstrictor Effect of Thromboxane A₂

By

JAN SVENSSON and BERTIL B. FRIEDHOLM

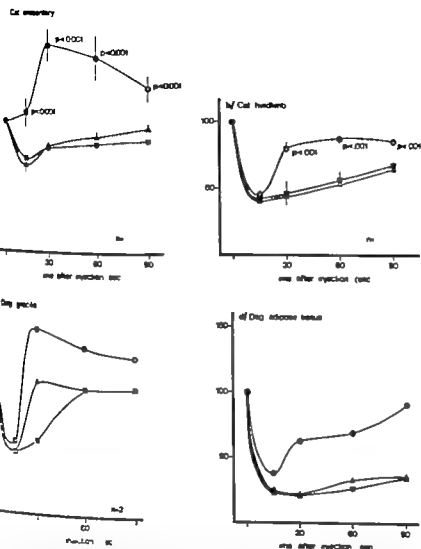
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Statistical comparisons were performed using Mann-Whitney U-test.

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Effect of platelet filtrate on the vascular resistance in a) cat mesentery b) cat hindlimb, c) dog gracilis d) dog adipose tissue. A washed platelet suspension was incubated with arachidonic acid as in methods. After filtration the platelet filtrate was incubated for 0-20 (O-O), 30-120 (L-Δ) min. The vascular resistance 60 sec after injection of the first (Tx_{A₂}-containing) as 96% higher in the cat mesentery, 60% higher in the cat hindlimb, 97% higher in the dog gracilis and 170% higher in the dog subcutaneous adipose tissue.

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the cat femoral bed. On the other hand PGH_2 (and PGG_2) sometimes produced a triphasic response (dilatation-constriction-dilatation) in the cat mesenteric circulation. Thus there is reason to assume that ADP present in platelets and released by arachidonic acid will contribute significantly to the vasoactivity of the platelet filtrate. In support of this contention is also the finding that ADP produces a larger vasodilatation in muscle and adipose tissue than in the mesenteric circulation in agreement with the findings with platelet filtrate.

In contrast to platelet filtrates that have been left for 90 s or more, filtrates injected immediately caused a significantly smaller degree of vasodilatation and in the case of the cat mesenteric circulation and the dog gracilis muscle actually caused a transient vasoconstriction. It is known that the platelet filtrates used contain 266 ± 46 ng TxA_2 /ml immediately after filtration (Hamberg and Svensson 1976). TxA_2 disappears with an apparent half time of 30–40 s (Hamberg *et al.* 1975). Thus significant amounts of TxA_2 would be present still in the unincubated platelet filtrates while the incubated platelet suspensions would contain 10 per cent of the original amount or less. Thus our findings suggest that filtrates of platelets aggregated with arachidonic acid contain two different types of vaso-activity: a stable vasodilatory material and an unstable vasoconstrictor substance. Since TxA_2 is present in the filtrates, and because it is quite unstable and causes constriction of isolated vascular smooth muscle the possibility is a strong one that the vasoconstrictor material is indeed TxA_2 .

Only in two tissues was the net effect of the vasodilatory and the vasoconstrictor material a decrease in flow and even in these it was moderate. This indicates that even though TxA_2 is a potent vasoconstrictor the simultaneous formation of vasodilator metabolites, i.e. AD and its breakdown products, may antagonize its actions. The net result may be only limited if any vasoconstriction. Furthermore, the present data indicate that the effect of materials released from the aggregated platelets may cause different effects on the circulation in different vascular beds.

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Impulse Dependent Adaptation in *Helix Pomatia* Neurons: Effect of the Impulse on the Firing Pattern

By

MORTEN COLDING-JØRGENSEN

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Abstract

COLDING-JØRGENSEN, M. *Impulse dependent adaptation in Helix pomatia neurons: Effect of the impulse on the firing pattern.* Acta physiol. scand. 1977 101 369-381

Adaptation of the ventral ganglion of *Helix pomatia* an adaptation type is demonstrated, where the impulse contributes to the adaptation, as if slowly changing outward current were associated to every spike. The current appears to be accumulated from impulse to impulse and the accumulated current to decay towards zero in the interval between the impulses. This type of adaptation is called *impulse dependent*. A method to distinguish between impulse dependent adaptation and impulse independent adaptation is described. Typical adaptation curves for the impulse dependent adaptation are shown. In strong adaptation there is linear relation between the steady state frequency and the applied current. When stimuli of short duration are applied repetitively neurons with impulse dependent adaptation respond with spikes on an increasing fraction of the stimulus as the stimulus strength is raised. A simplified model of the adaptation is proposed, and the firing pattern of the cell is compared with that of the model. In this way numerical values of the model parameters have been estimated.

Nerve cells and sensory cells are able to transform an outer action into a collection of action potentials, where the timing or rhythm of the action potentials in some way is related to the action. The mechanisms, which are responsible for this transformation—called the firing process, are, however, only sparsely known. A way of elucidating these mechanisms is to apply different, known stimuli to the nerve cell and to observe the resulting changes in the activity of the cell. Many nerve cells will thus start firing with a high firing frequency when a stimulus suddenly is applied to the cell, but the firing frequency decreases with time, when the stimulus is continued. This phenomenon is called adaptation.

While the mechanisms responsible for the nerve action potential itself have been studied intensely for the past 3 decades (Hodgkin and Huxley 1952, Dodge 1963, Frankenhaeuser and Huxley 1964, Connor and Stevens 1971), only a few attempts have been made to clarify the mechanisms responsible for the firing interval. Furthermore, since Hodgkin's (1952) description of the different adaptation types in *Carcinus* axons, most work on adaptation has been a phenomenological description of the firing behaviour (Eyzaguirre and others 1955, Forbes and Mantegazzini 1962, Iggo and Muir 1969, Nakajima and Oodera

the cat femoral bed. On the other hand PGH₂ (and PGG₂) sometimes produced a triphasic response (dilatation-constriction-dilatation) in the cat mesenteric circulation. This therefore is a reason to assume that ADP present in platelets and released by arachidonic acid will contribute significantly to the vasoactivity of the platelet filtrate. In support of this contention is also the finding that ADP produces a larger vasodilatation in muscle and adipose tissue than in the mesenteric circulation in agreement with the findings with platelet filtrate.

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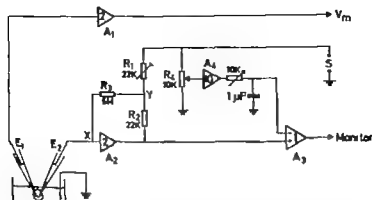


Fig. 1. Outline of the experimental setup. A_1 and A_2 are amplifiers with high input resistances, capacity time constants and gains of 2. A_4 is coupled as a current generator where the current, I_{cp} , through the resistor is proportional to the potential S as described in the text. The membrane potential is recorded by the amplifier A_2 , but when only one electrode is used the membrane potential is recorded by the amplifier A_1 .

potential directly to ground. With the potential S at zero R can be adjusted so no bias current is sent through the cell.

In some of the experiments only electrode E_2 was inserted into the cell. The membrane potential as then measured by amplifier A_2 here potential proportional to the current through the cell was obtained from the output of A_2 . By adjusting the resistor R_2 and slowing the output of A_4 little by the time constant of the RC-network the potential drop over the electrode could be cancelled out, at least when the current is of the order of a few nA or less.

The cells used in the experiments were selected at random, mainly on the surface of the posterior part of the ganglion.

Results

Demonstration of impulse dependency

In cells usually fired with a burst of activity when the electrodes penetrated the membrane. Some of the cells became silent, whereas others continued to fire with constant frequency. A hyperpolarizing or depolarizing current was led through the current electrode.

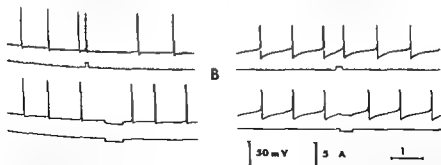


Fig. 2. Demonstration between cells with impulse dependent (A) and impulse independent (B) types of firing. For each cell is shown two recordings consisting of an upper trace displaying the intracellularly recorded membrane potential (monitor) and lower trace displaying the current passed through the electrode. Single electrode experiment.

1969 a, b Granit 1972, Connor 1975), distinguishing between fast and slow adaptation and between tonic and phasic behaviour. For the cat motoneurone, however it has been shown (Kernell and Sjöholm 1973, Baldissera and Gustafsson 1974, Gustafsson 1974) that the adaptation is accompanied by an increase in the potassium conductance, and that this increase can account for the adaptation of the neurone. Similar conductance changes have been demonstrated in several neurones (Jansen and Nichols 1973, Gola 1974, Barret and Barret 1976). Recently a model for the adaptation of neurones from *Archidoris montereyensis* and *Anisodoris mobilis* has been presented by Partridge and Stevens (1976), where the properties of the conductance were revealed through a voltage clamp technique.

In the present paper it is shown that the mechanisms governing the firing interval can be elucidated further by applying transient stimuli to the cell. In this way it is possible to distinguish between two apparently fundamentally different mechanisms. In one type of cells the firing interval seems to depend solely on *when* preceding impulses were elicited and not on *how* they were elicited. This type of behaviour is called *impulse dependent*. In another type of neurones the firing appears to be almost completely independent of the presence of preceding impulses. This firing is called *impulse independent* or since it in some way must be related to the stimulus itself *stimulus dependent*. The impulse dependent type is most easily understood if it is assumed that every impulse has the same effect on the firing period, that the effect is accumulated from impulse to impulse, but that it is limited in time. The effect may be due to an outward current associated to every impulse, and in the appendix a model is outlined, based on this possibility. By comparing the firing of the cell with the model important parameters of the cells are estimated. Preliminary reports have previously been given on the impulse dependent and stimulus dependent adaptation types (Colding-Jørgensen 1975 a, b, 1976 a).

Methods

The ventral ganglion of *Helix pomatia* was isolated, the covering connective tissue was removed, and the ganglion was placed in a chamber containing a solution of the following composition (mM): NaCl 10, KCl 4, CaCl₂ 7, MgCl₂ 3, Tris-Cl 5 (pH 7.5). Cells were impaled with glass microelectrodes filled with 3M KCl and with a resistance of 5-15 MΩ.

The experimental setup is shown in Fig. 1. Amplifier A₁ and A₂ are preamplifiers with high input resistance, capacity neutralisation, and with a gain of 2. The microelectrode E₁ was used to record the membrane potential of the cell, while the electrode E₂ was used to inject current through the cell membrane. The amplifier A₃ was used to control the current through the cell so it became independent of the resistance of the electrode E₂ and of the cell membrane potential. This can be illustrated in the following way. With the potential X at the input the output of A₃ is 2X. If the resistances of the resistor R₁ and R₂ are equal, and if the resistance of R₂ is much larger than R₁ and R₃, then the potential, Y, on the right hand side of R₃ will be

$$Y = \frac{1}{3}(S + 2X) = \frac{1}{3}S + \frac{2}{3}X,$$

where S is the potential applied at the terminal S. The current, I_m, through R₃ is therefore

$$I_m = (Y - X)/R = -S/2R,$$

which is independent of X. Since the input resistance of A₁ and A₂ is high, the current I_m must flow through the cell membrane to ground. With R₂ equal to 3 MΩ the current is 100 nA per volt applied at S. Further measurement of the current is not necessary and consequently the filling of the chamber

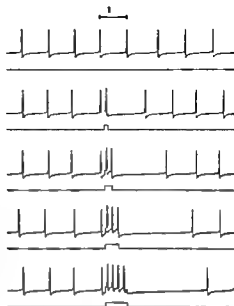


Fig. 4. Response of cell with impulse dependent system to an increasing number of impulses. In upper trace of each recording is the membrane potential (voltage) and the lower trace the current through the cell. Single electrode experiment.

through the cell, thereby eliciting 1, 2, 3, or 4 extra spikes. As the number of spikes was increased, the firing period just after the stimulation was prolonged more and more, but the period following the prolonged period was unaffected. The prolongation was least proportional to the number of spikes each spike increasing the firing period by 57 μ s. Impulse dependent adaptation is thus additive.

Response to step of constant current

In order to demonstrate the adaptation of the cells depolarizing current steps were passed through the membrane of the cell, and Fig. 5 shows the corresponding recordings of the membrane potential for steps of 0.2, 0.4 and 0.75 nA. The onset of the current can be seen as a small deflection from the resting potential in the beginning of each recording. The figure shows: (1) The adaptation occurs for small currents after the first few spikes thus the effect of a single impulse is strong. To a current of 0.2 nA the cell adapts completely after one spike. (2) The threshold potential does not change during the firing. The cell

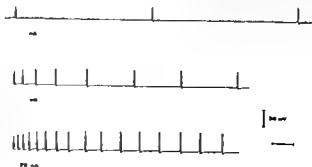


Fig. 5. Firing pattern of the impulse dependent type in response to current steps of different magnitude. Single electrode experiment. All two electrodes.

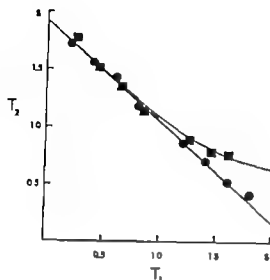


Fig. 3 Relation between the induced firing period T_1 and the subsequent firing period, T_2 as described in the text. \circ —cell 1 \square —cell 2. The curve is drawn according to eq. (6) in the appendix with the same parameters as for the curves in Fig. 2. Through the points for cell 1 is drawn a straight line.

to make the cell fire at a constant frequency usually 0.3–1 Hz. Some of the cells produced only phasic activity; these cells were not used. The cells were tested for impulse dependency as shown in Fig. 2. The two upper sets of recordings, A, are from one cell; the lower, B, from another cell. The current was adjusted to make the cells fire with a period between 1.0 and 1.1 s, and an additional rectangular current pulse was applied as shown on the figure. The two cells behaved differently. In A the introduction of an extra spike prolonged the subsequent period; the delay of the spike shortened it. In B only the period of the stimulus was affected. Thus the behaviour of the cell in A was modified by the presence of an impulse, the behaviour of the cell in B was not. The procedure distinguished reliably between impulse dependent and impulse independent behaviour and was therefore used to identify cells with impulse dependent adaptation.

Of the 147 cells investigated 81, or 55%, displayed impulse dependent firing, and 50–80% of these fired spontaneously. Stimulus dependent behaviour was found in 52 cells (35% of which 21 (14% of the total) displayed phasic activity. The remaining 14 cells (10%) showed only weak impulse dependency or no definite adaptation.

The impulse dependent behaviour can be elucidated further. With the stimulus applied at different times after a spike, thereby varying the duration, T , from the normal spike to the induced, the subsequent firing period, T_2 , depends on T . This is demonstrated in Fig. 3. The figure shows the relation between T and T_2 for two cells with impulse dependent adaptation. Each symbol represents an average of 5–15 samples with the same T and with a S.E. less than the size of the symbols. In all cases the mean value of the steady firing period, T , was 1.05 s (limits 1.0 and 1.1 s). For values of T less than T both cells gave a fairly linear relation between T and T_2 , but for T larger than T the two cells behaved in slightly different ways. Cell 1 (circles) continued to give a linear relation, whereas the relation for cell 2 (squares) seemed to approach a constant value for T_2 , which was rather large. It was not possible, even with very large values of T , to obtain a T_2 below 0.7 s.

Another way of demonstrating impulse dependency is illustrated in Fig. 4. The cell is firing with a frequency close to 10 Hz, when an additional rectangular current pulse is

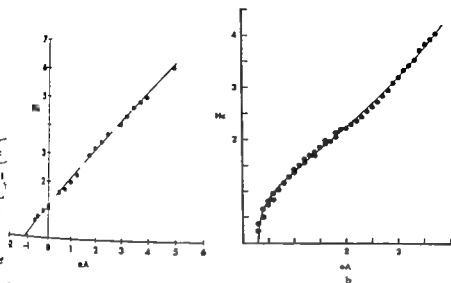


Fig. 7 Relation between the constant current stimulus and the resulting steady state firing frequency. Linear relation for cell with strong adaptation. b S-shaped relation for cell with weak adaptation. The current was driven according to eq. (4) of the appendix. Abscissa: current stimulus in nA, ordinate: firing frequency in Hz.

repeatedly occurring EPSP's the cells were stimulated by current pulses of 10-300 ms duration at a constant frequency of between 0.5 and 2 Hz. Such patterns of EPSP's was predominant in the ganglion. Driven in this manner the cell frequently failed to respond to large stimuli, and the failures became more frequent the faster the cell was driven, the smaller the stimulus strength, and the shorter the pulse duration. The behaviour is shown in Fig. 8 a. The cell was driven at a frequency of 1.75 Hz with current pulses of 10 ms duration and increasing stimulus strength. The cell responded with an increasing number of spikes as the current strength was increased, until it finally responded to every stimulus at a stimulus strength of 7 nA. The cell acted in this way as a frequency converter where the conversion ratio depended on the stimulus strength. In addition Fig. 8 a shows that even though the cell did not respond to every stimulus, its firing was very regular. The cell did not only respond to 33%, 50% or 67% of the stimuli. It responded on every third, every second or failed on every third respectively.

The behaviour may be understood by inspection of Fig. 8 b. The recording is from the same cell, but on a different time scale with a stimulus duration of 100 ms and an amplitude of 0.55 nA to enhance the effect. After a dropout the spike is delayed more and more with respect to the stimulus, as if the threshold were increasing during the firing. When the threshold becomes too large, the stimulus fails to excite the membrane, and a new dropout occurs. During this dropout (dropouts) the threshold decreases, until the stimulus again is sufficient to excite the membrane. The relation between the conversion ratio and the stimulus strength for the recordings in Fig. 8 is shown in Fig. 9. The number of spikes elicited per stimulus is plotted as a function of the stimulus strength. A straight line is drawn through the points indicating that the relation is fairly linear.

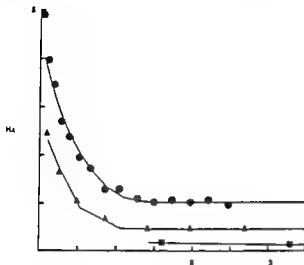


Fig. 6. Adaptation curves for the firing shown in Fig. 5. Abscissa: Time since the first spike in seconds, ordinate: instantaneous firing frequency in Hz. \blacksquare — \blacksquare 0.2 nA, \blacktriangle — \blacktriangle 0.4 nA and \bullet — \bullet 0.75 nA. The curves are drawn according to the model as given in equation (3) of the appendix.

fires, whenever the threshold is reached, even if the potential changes slowly (3) The firing is fairly regular even for long firing periods. The cells were able to fire steadily with firing periods as large as 5–15 s. (4) The amplitude of the action potentials increases in the beginning of each recording. This was frequently although not always, seen especially in cells, which had been hyperpolarized prior to the current step to prevent firing.

The instantaneous firing frequencies, the reciprocal of the period between a spike and its precedent, for the three recordings of Fig. 5 are plotted in Fig. 6 as functions of the time from the appearance of the first spike. The symbols represent the frequency measured from the recordings, and the curves are drawn according to the model (cf. the appendix). In the beginning of the current step the frequency is falling rather fast from a high value, whereafter it suddenly becomes constant. This "break adaptation" is typical for the impulse dependent firing, especially for small stimuli. In cells, where the adaptation was strong, the break could be very pronounced: the cell fired a few spikes close to each other at the onset of the stimulus, and subsequently the frequency fell to a steady value.

Thus, when the stimulus current is maintained the frequency becomes constant, and the relation between this steady state frequency and the current stimulus can be obtained. Such relations are shown in Fig. 7a and 7b. For a cell with a strong adaptation the relation is almost linear over a considerably large stimulus interval (Fig. 7a), whereas the relation for a cell with a weak adaptation is s-shaped (Fig. 7b). Notice that the cell in Fig. 7a was firing even when no current was passed through the cell and that it had to be hyperpolarized to prevent firing. Such spontaneous activity was frequently seen, but there seemed not to be any detectable difference in the behaviour of the active and silent cells apart from the spontaneous activity. Through the points of Fig. 7a is drawn a straight line with a slope of 1.0 Hz/nA, while the curve in Fig. 7b is drawn according to the model (cf. the appendix).

C. Response to repetitive stimulation

Neurons are normally excited by EPSPs (Excitatory postsynaptic potentials) acting as short current pulses either occurring repetitively or as single pulses. In order to imitate the

impulse through the current electrode. If the stimulus was too small or the delay too short a stimulus failed to excite the cell, and if the stimulus was too large or the delay too long, impulses would be triggered by every stimulus thereby giving rise to perpetual activity of the cell. However in some interval only a finite train of spikes would appear and at a constant value of the delay and the stimulus strength, the number of spikes elicited would be a constant too. In this way they could reliably multiply their firing frequency by given number, thus giving rise to conversion ratios larger than 1.

Discussion

Results seem to demonstrate that the presence or absence of an impulse can modify the firing pattern. This is most clearly demonstrated in Fig. 2 A. It is not likely that the stimulus itself should cause the behaviour because the pattern was independent of the strength and duration of the stimulus as long as only one impulse was elicited. Moreover, the same behaviour was observed in spontaneously firing cells, when small irregularities occurred in the firing rhythm, and also in these cases a linear relation between the firing period before (T_1) and after (T_2) the disturbance could be found. It is also likely that the cell was driven by a regular synaptic input. Such activity could drive the cell at a constant frequency but the frequency would be fixed. The firing frequency of the cell could, however easily be changed by passing a constant current through the cell, unless the cell was hyperpolarized to prevent firing, no regular synaptic activity could be detected. For the driven activity the relation between T_1 and T_2 would be linear but the slope should have been exactly -1 because the sum $T_1 + T_2$ should be equal to the steady firing period, T . The numerical value of the slope was, however always less than unity and it decreased, when T was increased.

Both cells (A and B of Fig. 2) displayed definite adaptation, so the mechanisms, which showed their repetitive behaviour must have been different. The experiments show that a cell with impulse dependent adaptation could act as a stable oscillator which could preserve an almost constant mean frequency in spite of disturbances. It was also able to display dynamic activity in response to a change in the stimulus. A rapid increase in the stimulus strength produced a large transient increase in the firing frequency but only a small increase in the steady frequency. In this way the cell was able to transmit information about transient phenomena and superimpose it on a stable firing. In contrast the cell in Fig. 2 B acted as an excellent follower which easily shifted its firing back and forth in response to a disturbance, but the firing period after the disturbance was unchanged.

The effect of an impulse on the firing seems to be additive, which means that the effect of one impulse is added to the effect of previous impulses (Fig. 4). The effect is, however transient in time (Fig. 2 and 4). After a disturbance the cell resumes its steady firing with the same firing period as before the disturbance.

Repetitive stimulation

In the experiment with repetitive stimulation the stimulus strength was changed, while the steady level of the current was unchanged. A similar firing pattern could have been obtained by using current pulses of the same size and then change the steady level of the

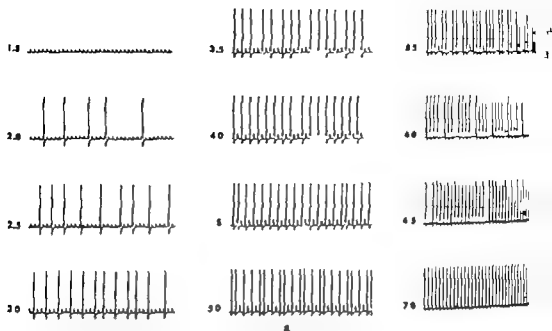
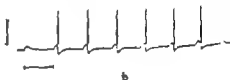


Fig. 8 Response to repetitive stimulation. a. Intracellular recordings from a cell with long-term adaptation stimulated with current pulses of 10 ms duration at a frequency of 1.75 Hz. At each record is shown the current strength in nA. (Two electrode experiment).

b. Same cell as in Fig. 8 a. The cell is stimulated with current pulses of 0.55 nA and with a duration of 100 ms. Potential scale: 50 mV time scale: 1 s.



By the presented procedure conversion ratios below 1 were found. The cell was able to fire with a frequency higher than the input frequency under some circumstances. This possibility was demonstrated in the following way. Every time a spike was elicited it triggered an electronic equipment which after a delay applied a rectangular current

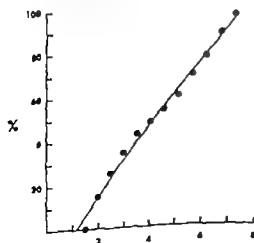


Fig. 9 Relation between the stimulus strength and the percentage of responses for the recordings shown in Fig. 8 a. Abscissa: Stimulus strength in nA and ordinate: Number of spikes elicited as percentage of the total number of stimuli applied. The straight line indicates that the relation is almost linear.

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current. The relation between the conversion ratio and the steady stimulus strength would then be the same as in Fig. 9 with the sum of the steady current and the current pulses as the abscissa. Within the ganglion the EPSP's do not change as the stimuli of Fig. 8a, but slowly changing synaptic inputs or changes in the composition of the extracellular fluid may act in the same way as changes in the steady current strength. Thus the cell with impulse-dependent adaptation is not only able to display the spatial and temporal summation of subthreshold stimuli but also to suppress the effect of suprathreshold stimuli.

The conversion ratio depends on the frequency of the current pulses. For small frequencies the ratio is 1 but for larger frequencies, where dropouts occur, the firing frequency becomes constant, as if the cell was stimulated by a constant current of the same strength as the pulses. If the size of the EPSP's is a constant, the cell should then fire at a rate which is independent of the frequency of the EPSP's. The conversion ratio may exceed 1, for example if an excitatory collateral from the axon is returning to the cell body either directly or through interposed neurones, and although the nature of such a collateral is completely speculative, its possible effect is interesting. Due to a constant propagation velocity and synaptic delay (possibly in interposed neurones) the total delay as well as the stimulating synaptic input would be constant. The conversion ratio could, however again, be varied by varying the basic, synaptic input to the cell.

The observed fraction of the impulse dependent cells (55%) in the ganglion was remarkably high and may not be representative for the whole ganglion. There might have been a tendency to search for impulse dependent cells at places, where they had been found previously and there may be differences between the superficial cells and the cells in the rest of the ganglion.

A model of adaptation

A possible explanation of the impulse dependent behaviour may be that the impulse is associated with an outward current, called the adaptation current, which tends to hyperpolarize the membrane and thus prolong the firing period. The current may be due to an increase in the potassium conductance or to an electrogenic pump as the most obvious possibilities, but in the simplified model of adaptation presented below nothing is assumed about the nature of the current.

The model has the following main features: 1. Whenever the membrane potential passes beyond the threshold, a spike is elicited. 2. The spike generating mechanism and the adaptation mechanism are mutually independent. 3. The adaptation is governed by an outward current, where each spike increases the current by the same amount, and the current decays exponentially towards zero with a large time constant. Finally the relation between the stimulating current, I_m , and the firing period T is assumed to be described by the following expression (Colding-Jørgensen 1976 b)

$$I_m - I = I \exp(-T/\tau_K) + I_A$$

where I is the rheobasic current, I_A is a constant and τ_K is the time constant for the potassium conductance participating in the spike formation.

It was found that a spike was triggered whenever a threshold was passed even when the potential changed slowly (Fig. 2, 4 and especially 5). This confirms the first assumption. The

average adaptation time constant of 100-2000 times the duration of the spike makes a distinction between a spike generating mechanism and an adaptation mechanism acceptable. The existence of an adaptation current with an exponential decay is not demonstrated in the present paper. It is only shown that the effect of an impulse is decaying towards zero with time, so the exponential decay can be taken as a simple, mathematical expression, which is able to fit the behaviour of the cells. However the assumption that each spike increases the adaptation current by the same amount seems to be justified by the demonstration in Fig. 4 and to some extent Fig. 7a.

It is interesting that the simple model is able to mimic the complex behaviour demonstrated in the previous sections. Furthermore, when the adaptation is strong and its time constant large, the equation above can be simplified to

$$I_m - I_0 = I_A$$

in order. Whenever the adaptation current, I_A , becomes smaller than the difference between the stimulating current, I_m , and the rheobasic current, I_0 , a new spike is elicited. With this simple expression and using eq. (2) of the appendix to find I_A most of the spike dependent behaviour can be understood. Only when the firing period according to this simplification approaches the refractory period of the cell (symbolized by the time constant in the appendix) a more detailed model has to be used. It can be seen from the expression, why the impulse dependent cell type is a stable oscillator even at low frequencies. The adaptation current offers a kind of feed-back control of the firing, which ensures that a new spike first is elicited, when I_A is decreased sufficiently no matter how small the difference $I_m - I_0$ is. The effect of a disturbance, which for example shortens the firing period, is then that the next firing period is prolonged in such a manner that the mean firing period almost is conserved.

The model shows that the current, I_A , released by a single spike is small. In Fig. 6 a value of $I_A = 0.04$ nA was used, and in Fig. 7a a value of 0.13 nA was found. If it is assumed that the current is due to an increase in the potassium conductance, the corresponding conductance can be found. The equilibrium potential for the potassium ion is in the order of 20 mV below the resting potential (cf. Colding-Jørgensen 1977) for the cell, so 0.13 nA corresponds to an increase in the potassium conductance of some $0.65 \cdot 10^{-6}$ S per spike, or to 10-20% of the resting value of the membrane conductance (resistance of the cell 10-30 MΩ). Thus, although the current of a single spike is small, few spikes fired in a row to each other can change the membrane conductance considerably.

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Appendix

The model

A thoroughly description of the model is given elsewhere (Colding-Jørgensen 1976 b). It has the following three main features. 1. Whenever the membrane potential passes beyond the threshold, an action potential or spike, is elicited. 2. It is possible to distinguish between a spike generating mechanism and an adaptation mechanism, which are mutually independent. 3. The adaptation is governed by an extra current, the adaptation current. Each spike increases this current by the same amount, I_A and the current decays exponentially towards zero with a large time constant τ_A .

According to the model the interval, T between two successive spikes is given by

$$I_m - I_0 = I \exp(-T/\tau_K) + I_A$$

where I_m is the transmembrane current, I_0 the rheobasic current, I is a constant, τ_K is the time constant for the normal potassium conductance participating in the spike formation, and I_A is the total adaptive current just before the next spike is elicited. If T_j is the duration of the j th firing period, the corresponding value of the adaptation current, I^j becomes

$$I^j = (I_A + I^j) \exp(-T_j/\tau_A)$$

according to the third assumption of the model.

When the membrane starts in a resting state and a current step, I_m , suddenly is passed through the membrane, the successive values of the firing period can be determined from eqns. (1) and (2) as

$$I_m - I = I \exp(-T_j/\tau_K) + (I_A + I_m - I_0 - I \exp(-T_{j-1}/\tau_K)) \exp(-T_j/\tau_A).$$

The value T for $j=1$ must then be set to infinity. This relation is used to draw the curves in Fig. 6. The parameters used are $I_0 = 0.16$ nA, $I = 2.6$ nA, $\tau_A = 6$ s, $\tau_K = 0.15$ s, and $\gamma = I_A/I = 0.04$.

When the cell is firing with a steady firing period, T eq. (3) reduces to

$$I_m - I_0 = I \exp(-T/\tau_K) + I_A \exp(-T/\tau_A)(1 - \exp(-T/\tau_A)).$$

For $\tau_K \ll T < \tau_A$ and with a not too small value of $\gamma = I_A/I$ eq. (4) can be approximated by

$$I_m - I_0 = I_A(\gamma \nu - \frac{1}{2})$$

where ν is the steady firing frequency. Such a linear relation between firing frequency and current is seen in Fig. 7 a, where the slope of the line is 1.6 Hz/nA. With a τ_A estimated for this cell of 7-8 s, eq. (5) gives I_A of 0.13 nA. I_0 can be found from the same equation to -1.08 nA. The negativity of I_0 corresponds to a spontaneously active cell. With respect to the cell of Fig. 7 b the relation between frequency and current is not linear. It can, however, be reproduced by the relation in eq. (4) (curved line of the figure) when the parameters are $I_0 = 0.3$ nA, $I = 6.9$ nA, $\tau_A = 10$ s, $\tau_K = 0.28$ s, and $\gamma = 0.002$. The constant γ is here very low. It corresponds to a I_A of 0.014 nA or ten times less than the estimate of I_A for the cell in Fig. 7 a.

In the case, where an extra spike is induced at time, T after spike I_A is, when the next spontaneous spike is elicited after a further time, T_p , found from eq. (2) to

$$I_A = (I_A + (I_A + I_A) \exp(-T_p/\tau_A)) \exp(-T/\tau_A)$$

where I_A is the value of I_A at the steady firing. Combining the equations (1), (7) and (4), the relation between T and T_p becomes

$$\exp(-T/\tau_K) + \gamma \exp(-T/\tau_A)(1 - \exp(-T/\tau_A)) = \exp(-T_p/\tau_K) + \gamma \exp(-(T + T_p)/\tau_A)(1 - \exp(-T/\tau_A)) + \gamma \exp(-T_p/\tau_A)$$

With the same approximations as in eq. (5), and for $T \ll 1.8T$ and $T < 0.25\tau_A$ the relation between T and T_p in eq. (6) is approximately linear with a slope of $-1/(1 + T/\tau_A)$. This is seen for cell 1 in Fig. 8. The slope of the line drawn through the points is 0.86, so according to the model the time constant τ_A should be 6.5 s. The relation for cell 2 is not linear but can be approximated, as shown on the figure, with the relation in eq. (6) with the same parameters as used in Fig. 6.

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Appendix

The model

A thoroughly description of the model is given elsewhere (Colding-Jørgensen 1976 b). It has the following three main features: 1. Whenever the membrane potential passes beyond the threshold, an action potential or spike, is elicited. 2. It is possible to distinguish between a spike generating mechanism and an adaptation mechanism, which are mutually independent. 3. The adaptation is governed by an extra current, the adaptation current. Each spike increases this current by the same amount, I_A , and the current decays exponentially towards zero with a large time constant τ_A .

According to the model the interval, T , between two successive spikes is given by

$$I_m - I_0 = I \exp(-T/\tau_K) + I_A$$

where I_m is the transmembraneal current, I_0 the rheobasic current, I is a constant, τ_K is the time constant for the normal potassium conductance participating in the spike formation, and I_A is the total adaptation current just before the next spike is elicited. If T_j is the duration of the j th firing period, the corresponding value of the adaptation current, I^j , becomes

$$I^j = (I_A + I^{j-1}) \exp(-T_j/\tau_A)$$

according to the third assumption of the model.

When the membrane starts in a resting state and a current step I_m , suddenly is passed through the membrane, the successive values of the firing period can be determined from eqns. (1) and (2) as

$$I_m - I_0 = I \exp(-T_j/\tau_K) + (I_A + I_m - I_0 - I \exp(-T_{j-1}/\tau_K)) \exp(-T_j/\tau_A).$$

The value T for $j=1$ must then be set to infinity. This relation is used to draw the curves in Fig. 6. The parameters used are $I = 0.16$ nA, $I_0 = 2.6$ nA, $\tau_A = 6$ s, $\tau_K = 0.15$ s, and $\gamma = I_A/I = 0.04$.

When the cell is firing with a steady firing period, T , eq. (3) reduces to

$$I_m - I_0 = I \exp(-T/\tau_K) + I_A \exp(-T/\tau_A) / (1 - \exp(-T/\tau_A)).$$

For $\tau_K \ll T < \tau_A$ and with a not too small value of $\gamma = I_A/I$, eq. (4) can be approximated by

$$I_m - I_0 = I_A (\gamma \tau_A - \frac{1}{f}),$$

where f is the steady firing frequency. Such a linear relation between firing frequency and current is seen in Fig. 7 a, where the slope of the line is 10 Hz/nA. With a τ_A estimated for this cell of 7-8 s, eq. (5) gives I_A of 0.13 nA. I_0 can be found from the same equation to -1.08 nA. The negativity of I_0 corresponds to a spontaneously active cell. With respect to the cell of Fig. 7 b the relation between frequency and current is not linear. It can, however, be reproduced by the relation in eq. (4) (curved line of the figure) when the parameters are $I = 0.3$ nA, $I_0 = 6.9$ nA, $\tau_A = 10$ s, $\tau_K = 0.28$ s, and $\gamma = 0.002$. The constant is here very low. It corresponds to an I_A of 0.014 nA or ten times less than the estimate of I_A for the cell in Fig. 7 a.

In the case, where an extra spike is induced at a time, T , after a spike, I_A is, when the next spontaneous spike is elicited after f rather time, T_0 , found from eq. (2) as

$$I_A = (I_A + (I_A + I_A) \exp(-T/\tau_A)) \exp(-T_0/\tau_A),$$

where I_A is the value of I_A at the steady firing. Combining the equations (1), (2) and (4), the relation between T and T_0 becomes

$$\exp(-T/\tau_K) + \gamma \exp(-T/\tau_A) / (1 - \exp(-T/\tau_A)) = \exp(-T_0/\tau_K) + \gamma \exp(-(T + T_0)/\tau_A) / (1 - \exp(-T/\tau_A)) + \gamma \exp(-T_0/\tau_A).$$

With the same approximations as in eq. (5), and for $T < 1.8\tau_A$ and $T_0 < 0.25\tau_A$ the relation between T and T_0 in eq. (6) is approximately linear with a slope of $-1/(1 + \gamma\tau_A)$. This is seen for cell 1 in Fig. 1. The slope of the line drawn through the points is 0.86, so according to the model the time constant τ_A should be 6.5 s. The relation for cell 2 is not linear but can be approximated, as shown on the figure, with the relation in eq. (6) with the same parameters as used in Fig. 6.

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Impulse Dependent Adaptation in *Helix Pomatia* Neurones: Demonstration of the Adaptation Conductance

By

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Abstract

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By conventional voltage clamp methods an increase in membrane conductance after a depolarizing pulse is demonstrated in neurones of *Helix pomatia*. This increase decays exponentially with a time constant in the range of 5-20 s. The rise of the conductance during the depolarization can be represented by 3 exponentials with time constants from 12 ms to 1 s. The steady state value of the conductance depends on the membrane potential in a sigmoid manner. The conductance gives rise to an outward current, which appears to be carried by potassium ions. The firing pattern of the cell is governed by the conductance. A short, rectangular potential change, a pulse clamp, is used to disturb the firing pattern of the fresh fire cell. The effect on the firing pattern depends on the height and duration of the clamp pulse in the same manner as the conductance increase. The normal firing produces similar changes in the membrane conductance.

An increase in membrane conductance following spike trains has been demonstrated in *Aplysia* (Brodwick and Junge 1972) in *Helix* (Gola 1974) in leech (Jansen and Nichol 1973), and in frog motoneurones (Barrett and Barrett 1976) while a slowly changing conductance has been shown to be responsible for the firing period in cat motoneurones (Baldwin and Gustafsson 1971, 1974; Kernell and Sjöholm 1973; Schwandt and Cahz 1973; Gustafsson 1974; Mauritz, Schlue, Richter and Nacimiento 1974). The repetitive behaviour of molluscan neurones has been modelled (Connor and Stevens 1971 a, b, c), but these cells displayed no or almost no adaptation. In a recent work, however, a slowly changing conductance has been demonstrated in the marine molluscs *Archidoris monterreui* and *Anisodoris nobilis* by Partridge and Stevens (1976). With this conductance included in a model of the snail neurone was able to display adaptation resembling the adaptive behaviour of the neurones. It was thus of interest to determine, whether the previously described impulse dependent firing in *Helix pomatia* neurones (Colding-Jørgensen 1975 a, b, 1976 b, 1977) was associated with a slowly decaying, outward current, and whether this current controlled the firing period as previously assumed (Colding-Jørgensen 1976 c, 1977).

methods were used in this investigation. The changes of the membrane conductance were studied with a conventional voltage clamp technique. But to examine whether the membrane conductance was responsible for the firing pattern of the cell another called the pulse clamp method, was used. A rectangular potential shift was imposed on the membrane, while the cell was firing freely. The effect on the firing period of the action potentials and amplitudes of the potential shift was then recorded.

Methods

Preparation of Helix pomatia—as isolated and the connective tissue removed. In some cases the tissue was softened with pronase-E applied as concentrated solution for 20 min and then washed. No difference in the behaviour was seen between treated and untreated ganglia. The ganglion was placed in a chamber containing solution of the following composition (mM): NaCl 80, KCl 5, $MgCl_2$ 5, Tris-Cl 5 (pH 7.5). The cells were impaled with two glass microelectrodes filled with KCl and with resistances of 5–15 M Ω .

Experimental setup—shown in Fig. 1 was described previously (Colding-Jørgensen 1977). The amplifier A_1 and the analogue switch AS. Amplifier A_1 and A_2 were preamplifiers with a resistance, capacity neutralization, and gain of 2. Amplifier A_3 was used to record the membrane potential, V_m . Amplifier A_4 was used to inject current into the cell. The current was either coming from the input S or from the output of the high gain amplifier A_5 depending on the position of the switch AS and its current of 100 nA per volt applied. The switch was graded by transistor logic (TTL) inputs with logical 1 close to 5 V and logical 0 close to 0 V and with the switch in a position, when C is low (close to 0 V). The setup then worked as a current clamp mode when controlled by S and with the membrane potential recorded by A_1 . When the input C is high (close to 5 V), the position of the switch is reversed, and the setup worked as a voltage clamp mode. The membrane potential was recorded by A_1 and fed into the amplifier A_6 together with the command V_c . The current through the cell was then controlled by the output of A_4 and by A_6 . The current was not measured directly but monitored at the output terminal I_m after passing through a variable resistor. The current used to neutralize the electrode capacity was therefore not included in the recordings, so the difference between the actual current through the cell and the current at I_m was small and depended mainly on the accuracy of the capacity neutralization. For slow changes in the current the difference was insignificant.

Current measurements. The cells were impaled by the current injecting electrode and clamped as described by Colding-Jørgensen (1977). Only cells with a definite response dependency were used. The recording electrode was then inserted and the analogue switch shifted, so the membrane potential was at the resting potential (45 to 60 mV) as preset on the input V. Conductance measurements were performed at the resting potential. Before and after conditioning pulses of 10 mV hyperpolarization and 100–200 ms duration were applied, and the current change during the 10 mV potential change was measured as the difference between the current just at the start of the pulse and the current just before the end of the pulse (Fig. 3). The conductance was calculated as the ratio between the measured current difference and the potential change of the pulse. The method should measure the effect of fast changes in the current trace related both to the changes in the membrane capacity and to fast, voltage dependent conductance changes. Small depolarizing potential changes of 1–2 mV produced the same results as the 10 mV hyperpolarization. After clamp. With the analogue switch in the current clamp position DC-current was passed to the cell and adjusted, so the cell fired with a constant frequency between 0.5 and 1 Hz. A TTL-pulse was fed to the terminal C at fixed time, T, after spikes switching the setup to the voltage clamp mode. A voltage clamp of short duration, called pulse clamp, was applied, where the clamp height was determined by the setting of V and the clamp duration by the duration of the TTL-pulse T. A rapid return in the voltage clamp, V, was preset so the output of A_4 equalled 5. When the clamp was on, V rose towards the desired value with a time constant of 0.5 to 1 ms. T accelerated the return to the resting potential, when the clamp was turned off, current pulse of 500 nA was added just at the clamp ended. By adjusting the duration of the pulse the membrane potential was brought back to 12.2 mV (Fig. 2).

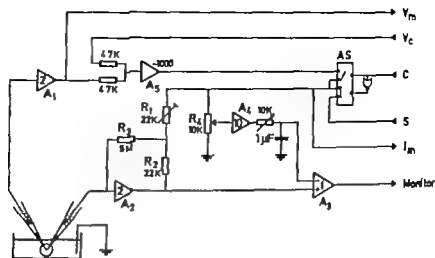


Fig. 1. Outline of the experimental setup.

The time T_p from the end of the pulse to the beginning of the next spontaneous spike is measured. During this period the membrane potential remained close to the threshold for several seconds (cf. Fig. 3). A small depolarizing disturbance could thus elicit a spike and shorten T considerably. The mean of the disturbed period, T_p , and the following period, T_s , should, however, be almost constant for a cell with strong impulse dependent adaptation (Colding-Jørgensen 1977), so T was found as

$$T = T' + T' - T$$

because T should have been equal to the steady firing period, T . For comparison the lowest T is divided by T because T could vary from time to time.

Reversed pulse clamp. The cell was clamped at the resting potential by setting the analogue switch, high mode. The input S was set to give a depolarizing current through the cell. A short TTL-pulse switch C to low mode and the current preset at S was passed through the now unclamped cell. By adjusting the duration of the pulse different numbers of spikes could be elicited before the pulse ended. When the clamp was switched on again, the membrane conductance was measured as previously described.

Results

A. Demonstration of the adaptation conductance

The adaptation conductance was demonstrated in the following way. The resting potential was determined and the membrane was clamped at this potential. The 10 mV pulses were added, and after some time the membrane potential was changed, usually in a depolarizing direction. This change was withheld for a short time, whereafter the membrane potential was returned to the resting value. An example of such an experiment is shown in Fig. 3. The upper trace shows the membrane potential, V_m , and the lower trace the transmembrane current, I_m . The membrane was at the beginning of the recording clamped at the resting

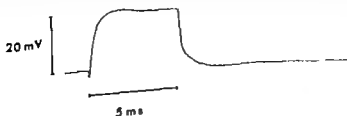


Fig. 2. Onset and offset of a 3 mV pulse clamp. Recording of the membrane potential during a pulse compensated pulse clamp.

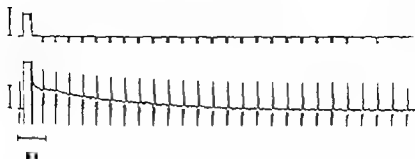


FIG. 3. Membrane potential and current during voltage clamps. Upper trace membrane potential, lower trace current. 10 mV hyperpolarization every 12 s. In the beginning of the recording 40 mV depolarization.

(-45 mV) and 10 mV hyperpolarizing pulses of 200 ms duration were imposed 12 s. Shortly after the start of the recording the membrane was depolarized 40 mV and repolarized to the resting potential after 1.8 s. During the depolarization the recorder was overloaded, but after the repolarization an outward current is seen, which decays towards zero. The current change corresponding to the 10 mV pulses is the depolarization larger than before the depolarization, and also this increase slowly with time. In Fig. 4 are shown the current (in nA, circles) and the difference between the conductance and the resting conductance (squares) as measured by the 10 mV pulses of 10^{-4} mho on a logarithmic scale as functions of the time after the end of depolarization. Both the current and the excess conductance decayed exponentially to zero. By linear regression the time constant for the decay was found to be 16.8 s for current and 17.1 for the conductance, so the current and the conductance can be taken with the same time constant of 17 s. This parallel decay implies that the current is proportional to the excess conductance—the factor of proportionality being 20.3 mV in earlier experiments with the same cell, but with different height and duration of the depolarization, yielded time constants close to 17 s and factors of proportionality close to

20 mV. A reasonable conclusion that the slowly decaying current, triggered by the 40 mV depolarization, was due to an increase in the membrane conductance, which declined exponentially with a time constant of 17 s. The ratio of 20 mV between the current and the conductance may correspond to the difference between the resting membrane potential and the equilibrium potential for the ion (ions) carrying the current. With the resting potential at -65 mV the equilibrium potential for the ion was then estimated to -45 mV, 20 mV above the resting potential. Different cells gave by this method equilibrium potentials between 60 and 70 mV. In some experiments the equilibrium potential was estimated from the reversal potential for the slowly changing current. This gave similar results.

In particular cell the conductance decayed with a time constant in the interval 5–20 s, depending on the amplitude and duration of the depolarization, as long as the repolarization was performed to the same potential. The conductance after the depolarization decayed, however, with the duration and the amplitude of the depolarization. The

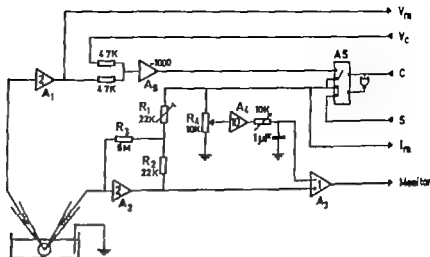


Fig. 1. Outline of the experimental setup.

The time, T_0 , from the end of the pulse to the beginning of the next spontaneous spike was measured. During this period the membrane potential remained close to the threshold for several seconds (cf. Fig. 4). A small depolarizing disturbance could thus elicit a spike and shorten T_0 considerably. The sum of the disturbed period T_0' and the following period, T_0'' , should, however, be almost constant for a cell showing impulse dependent adaptation (Colding-Jørgensen 1977), so T was found as

$$T = T_0' + T_0'' - T$$

because T_0'' should have been equal to the steady firing period, T . For comparison the found T_0' is divided by T because T could vary from time to time.

Reversed pulse clamp. The cell was clamped at the resting potential by setting the analogue switch C to high mode. The input S was set to give a depolarizing current through the cell. A short TTL-pulse switched C to low mode and the current preset at S was passed through the now unclamped cell. By adjusting the duration of the pulse different numbers of spikes could be elicited before the pulse ended. When the clamp was switched on again, the membrane conductance was measured as previously described.

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The adaptation conductance was demonstrated in the following way: The resting potential was determined and the membrane was clamped at this potential. The 10 mV pulses were added and after some time the membrane potential was changed, usually in a depolarizing direction. This change was withheld for a short time, whereafter the membrane potential returned to the resting value. An example of such an experiment is shown in Fig. 3. The upper trace shows the membrane potential V_m and the lower trace the transmembrane current, I_m . The membrane was at the beginning of the recording clamped at the resting

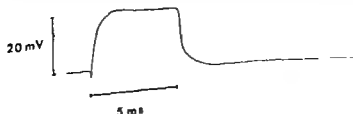


Fig. 3. Onset and offset of a 10 mV pulse clamp. Recording of membrane potential during a compensated pulse clamp.

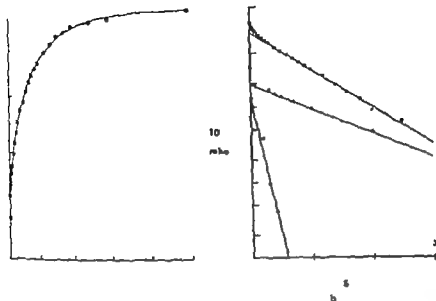


Fig. 5. Relation between the duration of -40 mV depolarization and the conductance just after the end of the depolarization. Abscissa: Duration of the pulse in s, ordinate: Total conductance in units of 10^{-10} mho. The curve drawn according to equation (1) is the solid.

Fig. 6. Resolution of the relation in Fig. 5 as three exponentials. Abscissa: duration of the clamp (s) on different scales. Ordinate: Difference between the maximal conductance and the conductance at t s (lower, linear scale) together with the two remaining components on different time scale (logarithmic and squares, upper scale).

which showed relation between the steady state conductance and the potential with only 1 per cent of the conductance present at the resting potential.

The relation is more curved for small conductances than for large, so the frequently used formula of the type $y = 1/(1 + e^{-x})$ does not fit the data very well. The data can, however, be approximated well by a logarithmic error function. The smooth curve in Fig. 6 is drawn according to the equation

$$g = 0.9 \cdot 10^{-9} (\operatorname{erf}(x) + 1) \quad (2)$$

with g in mho and with

$$x = \ln \frac{V + 65}{48}$$

where V is the membrane potential in units of mV

The influence of the conductance on the firing pattern of the cell

The result of a pulse clamp experiment is shown in the upper trace of Fig. 7. The cell was firing freely when a pulse of 55 mV depolarization for 60 ms was applied shortly after a spike. After the pulse the cell was silent for some time, until it resumed the steady firing. In the lower trace the firing was interrupted by two pulse clamps of the same height as

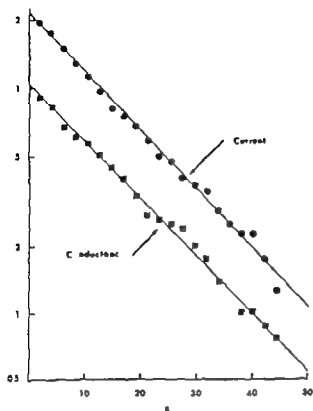


Fig. 4 Decay of membrane current and conductance after a depolarization of 40 mV/2.5 s. Abscissa: Time (s) after the end of depolarization. Ordinate: Membrane current (circles) in pA and membrane conductance minus resting conductance (squares) in units of 10^{-7} mho.

relation between the duration of the depolarization and the conductance just after the end of the depolarization was found by depolarizing the membrane 40 mV for durations between 20 ms and 5 s and extrapolating the conductance from the decay pattern. The conductance then rose from the resting value of $0.25 \cdot 10^{-7}$ mho to a maximum of $1.5 \cdot 10^{-7}$ mho (Fig. 5a), but the rise did not follow a single exponential function. The difference between the conductance of Fig. 5a and the maximal conductance is plotted in Fig. 5b on a logarithmic scale (upper curve, lower time scale). With an exponential peeling off technique (Rapp, 1967) three exponentials could be extracted with time constants of 0.89 s, 0.14 s, and 0.012 s and with intercepts of 0.93, 0.19 and 0.13 times 10^{-7} mho. Hence, the rise of the conductance g , is for a depolarization of 40 mV given by

$$g = 1.5 - 0.13 \exp(-D/0.012) - 0.19 \exp(-D/0.14) - 0.93 \exp(-D/0.89) \quad (1)$$

in units of 10^{-7} mho and with D as the duration of the depolarization. The solid curve in Fig. 5a is drawn according to equation (1).

The relation between the steady state conductance and the membrane potential is shown in Fig. 6. In order to avoid contributions from other currents the measurements were made as the difference between the conductance just after and just before a potential change of 3 s duration to the value shown on the abscissa (resting potential -50 mV). For hyperpolarizations below -65 mV no further decrease in conductance was seen, so the resting conductance was assumed to be zero at -65 mV. Measurements given

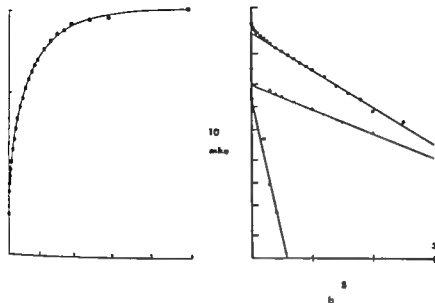


Fig. 5. Relation between the duration of 40 mV depolarization and the conductance just after the end of the depolarization. Abscissa: Duration of the pulse in s, ordinate: Total conductance in units of 10^{-10} mho. The curve is drawn according to equation (1) in the text.

Fig. 6. Examples of the relation in Fig. 5 in three exponentials. Abscissa: duration of the clamp (s) on different scales. Ordinate: Difference between the maximal conductance and the conductance in Fig. 5 (circles, lower scale) together with the two remaining components on different time scale (squares and squares, upper scale).

with sigmoid relation between the steady state conductance and the potential with only 1 per cent of the conductance present at the resting potential.

The relation is more curved for small conductances than for large, so the frequently used formula of the type $y = 1/(1 + e^{-x})$ does not fit the data very well. The data can, however, be approximated well by a logarithmic error function. The smooth curve in Fig. 6 is drawn according to the equation

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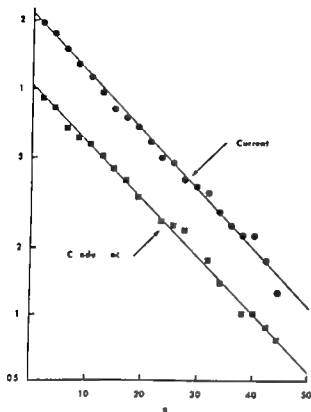


Fig. 4 Decay of membrane current and conductance after a depolarization of 40 mV 2.5 s. Abscissa: Time (s) after the end of depolarization. Ordinate: Membrane current (circles) in nA and membrane conductance minus resting conductance (squares) in 10^{-6} mho.

relation between the duration of the depolarization and the conductance just after the end of the depolarization was found by depolarizing the membrane 40 mV for durations between 20 ms and 5 s and extrapolating the conductance from the decay pattern. The conductance then rose from the resting value of $0.25 \cdot 10^{-6}$ mho to a maximum of $1.5 \cdot 10^{-6}$ mho (Fig. 5a) but the rise did not follow a single exponential function. The difference between the conductance of Fig. 5a and the maximal conductance is plotted in Fig. 5b on a logarithmic scale (upper curve, lower time scale). With an exponential peeling off technique (Rigby, 1967) three exponentials could be extracted with time constants of 0.89 s, 0.14 s, and 0.012 s and with intercepts of 0.93, 0.19 and 0.13 times 10^{-6} mho. Hence, the rise of the conductance g for a depolarization of 40 mV given by

$$g = 1.5 - 0.13 \exp(-D/0.012) - 0.19 \exp(-D/0.14) - 0.93 \exp(-D/0.89) \quad (1)$$

In units of 10^{-6} mho and with D as the duration of the depolarization. The solid curve in Fig. 5a is drawn according to equation (1).

The relation between the steady state conductance and the membrane potential is shown in Fig. 6. In order to avoid contributions from other currents the measurements were made as the difference between the conductance just after and just before a potential change of 3 s duration to the value shown on the abscissa (resting potential -50 mV). For hyperpolarizations below -65 mV no further decrease in conductance was seen, so the adaptation conductance was assumed to be zero at -65 mV. The measurements gave

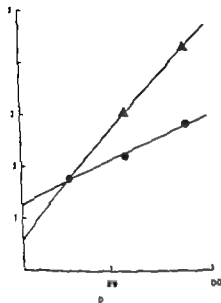


Fig. 8

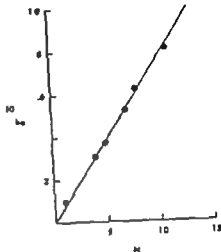


Fig. 9

Fig. 8. Relation between the total clamp duration and T_p/T for single clamps (circles) and multiple clamps (triangles). The axes are defined in the text.

Fig. 9. Relation between the number of spikes elicited during reverse pulse clamp and the increase in membrane conductance produced by the spikes. Abscissa: Number N , of spikes. Ordinate: Membrane conductance just after the pulse minus the resting conductance.

the, for the multiple pulses. The intercepts with the ordinate are 1.24 and 0.57 respectively.

For two and three pulses T_p/T is much larger than the corresponding value for single pulses using 10 and 90 ms. The intercept with the ordinate of 0.57 corresponds to the value of T_p/T for no pulse, or to 1 T_p/T where T is the period between the spikes before the pulse. The value of T_p/T was 0.33 to 0.4 so the intercept of 0.57 corresponds fairly well to the mean value of T_p/T for no pulse. The number of pulses and their effect on T is proportional, and the effect therefore additive—at least for small numbers.

During a pulse clamp an increase in conductance may be triggered as described in section A. The magnitude of this increase during the different pulses, calculated from eq (1), is reported in table 1 with the prolongation of the silent period as shown in Fig. 8. It appears from the last column that the ratio between the prolongation of the silent period and the conductance is fairly constant with mean of $6.7 \cdot 10^{-4}$ mho. There are thus a good correlation between the conductance increase and its effect on the firing pattern, so it can be concluded that the conductance demonstrated in section A may be controlling the firing period of the cell.

To examine whether the spontaneous firing of the cell produced a conductance change as demonstrated in section A, a reverse pulse clamp was used. The result is illustrated in Fig. 9.

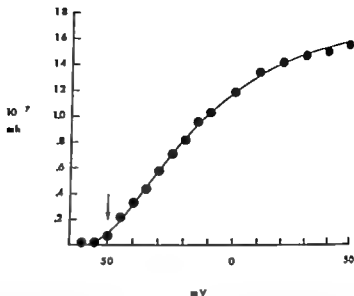


Fig. 6. Dependence of the adaptation conductance on the depolarization height. Abscissa: Depolarization from the resting potential in mV. Ordinate: The conductance just after the depolarization minus minimal value of the conductance. The resting potential is indicated by an arrow. The solid curve drawn according to eq. (2) in the text.

before, but with a duration of 30 ms and an interval of 70 ms. The silent period was much longer in the case of two pulses of 30 ms each than in the case with one pulse of 60 ms. This phenomenon illustrates that the mechanisms responsible for the effect of the pulse on the firing pattern has a rather complicated time dependency corresponding to the time dependency of the conductance demonstrated in section A.

The result of a series of such experiments with a depolarization of 45 mV and duration of multiples of 30 ms is shown in Fig. 8. Each symbol represents an average of 5-15 samples corrected as described under methods and with a S.E. of the same size as the symbols. The abscissa is the duration of a single pulse (circles) of 30, 60 or 90 ms or the total duration of 1, 2 or 3 pulses (triangles) of 30 ms each. The ordinate is the duration of the silent period after the pulse(s) relative to the steady firing period. The two sets of symbols form straight lines with slopes of 16.7 s⁻¹ for the single pulses and 40.6 s⁻¹ or 1.2 p.

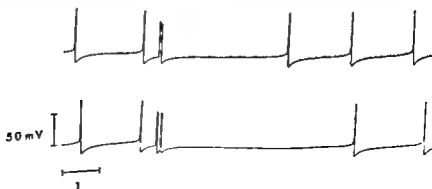


Fig. 7. Single and double pulse clamp. The upper trace shows the effect of a single pulse clamp of 60 ms. The lower trace shows the effect of two clamps of 30 ms each and with an interval of 70 ms.

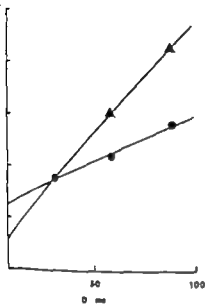


Fig. 8

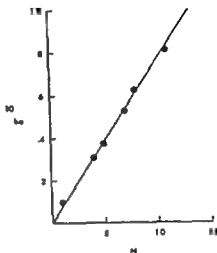


Fig. 9

Relation between the total clamp duration and T_p/T for single clamps (circles) and multiple clamps (triangles).

Relation between the number of spikes elicited during reverse pulse clamp and the increase in membrane conductance released by the spikes. Abscissa: Number N , of spikes. Ordinate: Membrane conductance just after the pulse minus the resting conductance.

for the multiple pulses. The intercepts with the ordinate are 1.24 and 0.57 respec-

two and three pulses T_p/T is much larger than the corresponding values for single pulses of 60 and 90 ms. The intercept with the ordinate of 0.57 corresponds to the value of for no pulse, or to $1 - T_p/T$ where T is the period between the spikes before the pulse for no pulse. The value of T_p/T was 0.33 to 0.4, so the intercept of 0.57 corresponds fairly to the mean value of T_p/T for no pulse. The number of pulses and their effect on T is proportional, and the effect therefore additive—at least for small numbers.

During a pulse clamp an increase in conductance may be triggered as described in section A. The magnitude of this increase during the different pulses, calculated from eq. (1), compared in table 1 with the prolongation of the silent period as shown in Fig. 8. It can be seen from the last column that the ratio between the prolongation of the silent period and the conductance is fairly constant with a mean of $6.7 \cdot 10^{-7}$ mho $^{-1}$. There are thus a close correlation between the conductance increase and its effect on the firing pattern, so it can be concluded that the conductance demonstrated in section A may be controlling the firing period of the cell.

To examine whether the spontaneous firing of the cell produced a conductance change as indicated in section A, a reverse pulse clamp was used. The result is illustrated in Fig. 9

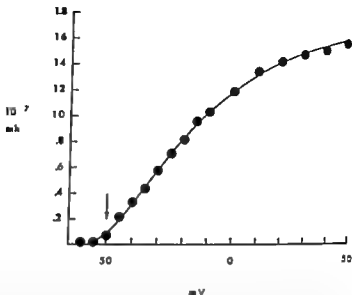


Fig. 6. Dependence of the adaptation conductance on the depolarization height. Abscissa: Depolarization from the resting potential in mV. Ordinate: The conductance just after the depolarization minus minimal value of the conductance. The resting potential is indicated by an arrow. The solid curve drawn according to eq. (2) in the text.

before, but with a duration of 30 ms and an interval of 70 ms. The silent period was longer in the case of two pulses of 30 ms each than in the case with one pulse of 60 ms. This phenomenon illustrates that the mechanisms responsible for the effect of the pulse on the firing pattern has a rather complicated time dependency corresponding to the time dependency of the conductance demonstrated in section A.

The result of a series of such experiments with a depolarization of 45 mV and duration of multiples of 30 ms is shown in Fig. 8. Each symbol represents an average of 5–15 samples corrected as described under methods and with a S.E. of the same size as the symbols. The abscissa is the duration of a single pulse (circles) of 30, 60 or 90 ms or the total duration of 1, 2 or 3 pulses (triangles) of 30 ms each. The ordinate is the duration of the silent period after the pulse(s) relative to the steady firing period. The two sets of symbols form straight lines with slopes of 16.7 s^{-1} for the single pulses and 40.6 s^{-1} or 12.2 s^{-1} for the double pulses.

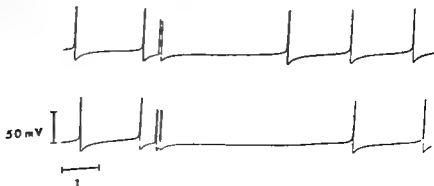


Fig. 7. Single and double pulse clamp. The upper trace shows the effect of single pulse clamp of 60 ms. The lower trace shows the effect of two clamps of 30 ms each and with an interval of 70 ms.

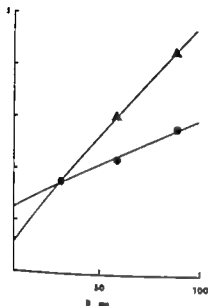


Fig. 8

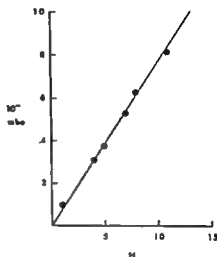


Fig. 9

Relation between the total clamp duration and T_p/T for single clamps (circles) and multiple clamps (triangles).

Relation between the number of spikes elicited during reverse pulse clamp and the increase in membrane conductance elicited by the spikes. Abcissa: Number, N , of spikes. Ordinate: Membrane conductance just after the pulse minus the resting conductance.

for the multiple pulses. The intercepts with the ordinate are 1.4 and 0.57 respec-

tively and three pulses T_p/T is much larger than the corresponding value for single pulses of 60 and 90 ms. The intercept with the ordinate of 0.57 corresponds to the value of T_p/T for no pulse, or to $1/T$ where T is the period between the spike before the pulse and the spike after the pulse. The value of T_p/T was 0.33 to 0.4, so the intercept of 0.57 corresponds fairly well to the mean value of T_p/T for no pulse. The number of pulses and their effect on T is proportional, and the effect therefore additive—at least for small numbers.

The magnitude of this increase during the different pulses, calculated from eq. (1), is compared in table 1 with the prolongation of the silent period as shown in Fig. 8. It is seen from the last column that the ratio between the prolongation of the silent period and the conductance increase is fairly constant with a mean of $6.7 \cdot 10^{-6}$ mho. There are thus a correlation between the conductance increase and its effect on the firing pattern, so it can be concluded that the conductance demonstrated in section A may be controlling the firing period of the cell.

It remains to be determined whether the spontaneous firing of the cell produced a conductance change as was observed in section A when a reverse pulse clamp was used. The result is illustrated in Fig. 9

TABLE I Comparison between the relative prolongation of the silent period after pulse clamps and conductance increase calculated for similar clamps from eq. (1).

Pulse duration ms	Relative prolongation of the silent period $T_s/T - 0.57$	Calculated conductance increase 10^{-4} mho	Prolongation per mho 10^3 mho $^{-1}$
30	1.20	0.18	6.7
60	1.61	0.25	6.4
90	2.20	0.31	7.1
30	2.43	0.36	6.8
3 30	3.60	0.54	6.7

which shows that the conductance increased proportional to the number of spikes to $0.76 \cdot 10^{-4}$ mho per spike. This conductance increase per spike can be compared with the value estimated from eq. (1). The half value of the depolarization for a spike from a neuron was approximately 40 mV and its duration 9 ms. According to eq. (1) the conductance (the value of g minus the resting conductance) corresponding to 9 ms is $0.18 \cdot 10^{-4}$ mho. The effect of a single spike should therefore be of that order of magnitude.

Discussion

The main result of this investigation is that the membrane current and the change in membrane conductance displayed almost identical decay patterns after the membrane had been depolarized by 40 mV and then repolarized (Fig. 4). This strongly supports the concept that the calculated conductance changes are real and makes it unlikely that the current after the end of the depolarization is produced by an electrogenic pump as found by Sokolove and Cooke (1971) for the tonic crayfish stretch receptor and by Jansen and Nicolls (1973) in leech neurones. It was not possible to measure the increase of the adaptation conductance during the depolarization itself due to simultaneous changes in other conductance components. All the cells displayed a marked inactivation of the outward current (up to 90% inactivation at large depolarizations), and the adaptation current could then only be seen at small depolarizations, where the outward current rose again after the initial decline. The initial current, measured 100 ms after the beginning of a depolarization, was thus smaller than the steady state current (after 5 s) for small depolarizations, but became larger than the steady state current for depolarizations larger than 70–40 mV. The rise of the conductance was therefore found indirectly as shown in Fig. 5. By this method it was invariably found that the rise was much faster than the decay. Normally at least two time constants could be resolved: one in the interval 0.7–1.0 s and another in the interval 0.1–0.3 s. The effect of the fast component in eq. (1) was then seen as an apparent increase in the conductance extrapolated to zero duration of the clamp.

Thus the small time constant of 12 ms obtained for the residual fast component may be accurate, because the change in the conductance for small durations approaches the limit of the method. The time constant is, however, of interest, because it is of the same order of magnitude as the duration of the spike, and the fast component may therefore be the main cause for the conductance increase during a single spike. This in-

from eq (1) and from the pulse clamp method found to be approximately $0.8 \cdot 10^{-4}$ mho, which agrees well with the $0.65 \cdot 10^{-4}$ mho estimated for a somewhat smaller cell by the method (Colding-Jørgensen 1977).

The pulse clamp as a reliable method to examine the effect of the adaptation conductance on the firing pattern. It was, however, performed with a fully adapted membrane with an small adaptation conductance, whereas the voltage clamp was performed from a resting membrane, here the adaptation conductance was small. The significance of this is not clear. The increased conductance before a pulse clamp might result in a larger increase during the pulse, but this effect was not observed. In the experiment with multiple pulse clamps the increase in firing period was still proportional to the number of pulses, even with the tripple pulses, where the firing period became more than four times the steady state value. The effect on the firing pattern of a certain change in conductance was to be fairly constant. From table 1 the relative prolongation of the firing period is 10^{-4} mho $^{-1}$ and in a previous paper (Colding-Jørgensen 1977) the conductance change of $0.65 \cdot 10^{-4}$ mho during a single spike gave a relative increase in the firing period of 0.57 corresponding to a ratio of $9 \cdot 10^4$ mho.

The linear relation between T_d/T and the duration, D found in Fig. 8 is at first sight in agreement with eq (1). However for durations within the interval 30–100 ms the relation in eq. (1) is almost linear with a slope of $2 \cdot 10^{-4}$ mho/s. With the ratio found in eq. (1) this should correspond to a slope for the line through the circles in Fig. 8 of 13.4 s^{-1} which is close to the found 16.7 s^{-1} .

The results presented in section A differ from the adaptation of the molluscan neurones studied by Partridge and Stevens (1976) in several respects. They assumed that the time constant for the rise of the conductance was the same as for the decay. As seen from Fig. 5 eq (1) this is definitely not the case for the impulse dependent adaptation in *Helix*. The time constant of the conductance contains time constants 3 orders of magnitude smaller than the time constant of the decay, and the time dependence of the conductance can therefore not be described by first order kinetics. Moreover an activation of the conductance depending on a higher order variable following first order kinetics will not apply either because it would give an activation which were slower than the offset.

In this respect the steady state value of the conductance at different membrane potentials is also different. Partridge and Stevens (1976) found that the conductance could be approximated by a symmetrical, sigmoid curve with a half maximum value around -1 mV and which were almost maximal at the resting potential whereas the present results give an asymmetrical curve with only 5 per cent of the maximal value present at the resting potential (Fig. 6). There are thus marked differences between the adaptation behaviour of the two cell types. While the type presented in the previous sections definitely displays an impulse dependent behaviour the cells presented by Partridge and Stevens (1976) seem to be of another type, which has been called stimulus dependent (Colding-Jørgensen 1976). It has also been demonstrated in *Helix* neurones (Colding-Jørgensen 1976 b).

The nature of the adaptation conductance is not clear but it has been shown that the entry of calcium ions through the membrane has a marked influence on the conductance. It is most probably the potassium conductance (Baker, Hodgkin and

Ridgeway 1971 Feltz, Krnjević and Lisiewicz 1972 Meech 1972, 1974 Kostyal, Krisz and Doroshenko 1974 Meech and Standen 1975 Vassort 1975 Heyer and Lux 1974 Furthermore, the decay of membrane conductance of *Aplysia* and *Helix aspersa* after calcium injection seems to contain time constants of approximately the same magnitude as the time constant for the adaptation conductance found in the present study (Meech 1972, 1974)

The increase in potassium conductance following a depolarization may thus be a result of a calcium influx, and the complex time dependency of the conductance may thus reflect the movement of calcium ions during and after the depolarization. In this way the fast component of the conductance could be due to a rapid calcium influx passing through the same pathway as during the normal spike.

The fundamental assumption for the impulse dependent adaptation is that each impulse gives rise to a constant amount of adaptation current. This has been shown to be the case for *Helix* neurones. The current can be due to an electrogenic pump or an increased potassium conductance as the most obvious possibilities. The present findings demonstrate that the membrane conductance increases during the adaptation and that the ion in question most probably is the potassium ion, because the estimated equilibrium potential for the adaptation current of -60 to -70 mV corresponds to the equilibrium potential for the potassium ion in snails (Connor and Stevens 1971 Neher 1971 Meech 1974 Goss 1974 Meech and Standen 1975 Partridge and Stevens 1976)

In the model (Colding Jørgensen 1976 c) a simple time dependency was assumed, and the steady state conductance was assumed to be very low for potentials near the resting potential. The impulse dependency was then brought forward by assuming that the adaptation conductance only became finite during a spike and decayed towards zero between the spikes. The present findings disclose another type of impulse dependency. The conductance becomes finite already at small depolarizations, but during a fast potential change—as during a spike—the fast onset of the conductance results in a rapid increase in conductance, while the slow decay in the interval between the spikes is responsible for the accumulation of the conductance increase.

Both types do however give rise to an almost constant amount of adaptation current per spike, so their behaviour during repetitive firing can be assumed to be almost identical.

A complete mathematical description of the kinetics of the adaptation conductance is not possible on basis of the present findings. The onset of the conductance is only given for depolarizations of 40 mV and the offset only at the resting potential. Depolarizations to different potentials yield similar results, but further experiments are needed in order to make it possible to give a quantitative description of the adaptation conductance.

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Angiotensin II Intratubular and Renal Capillary Pressure Regulation in the Rat Kidney

By

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Abstract

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Experiments were designed to test the hypothesis that angiotensin II, in its action on receptor sites vascular smooth muscle and the proximal tubule, acts homeostatically to stabilize hydrostatic pressure in the proximal tubule, and as a consequence, maintains a constant flow of fluid to the distal tubule. Infusion at 20 nl min⁻¹ of Ileu⁸ angiotensin II [(Ileu⁸)-A II] (20 g ml⁻¹) into peritubular capillaries adjacent to proximal tubule was found to be accompanied by an increase in hydrostatic pressure in both the intra capillaries and the proximal tubule and by a decrease in the rate of proximal fluid reabsorption (RPFR). RPFR was derived from timed quantitative collections of tubular fluid from the last superficial proximal segments. Tubular pressure before infusion and pressures in noninfused capillaries were not related to plasma renin activity (PRA). 1-Sarcosine, 8-alanine-angiotensin II [(Sar¹ Ala⁸)-A II] is known to be competitive antagonist to the action of angiotensin II on vascular smooth muscle and on adrenocortical zona glomerulosa cells. Infusion, at 20 nl min⁻¹ of (Sar¹ Ala⁸)-A II (333 ng ml⁻¹) into peritubular capillaries caused a decrease in hydrostatic pressure in the peritubular capillaries and adjacent proximal tubule. The most striking fall in pressure (-4 cmH₂O) occurred in rat with high levels of PRA. No pressure change resulted from infusion with a mixture of (Ileu⁸)-A II and (Sar¹ Ala⁸)-A II. (Sar¹ Ala⁸)-A II in this mixture blocked the inhibitory effect of (Ileu⁸)-A II on RPFR, but infusion of the angiotensin antagonist alone did not influence RPFR. It is concluded that angiotensin II is physiological regulator of hydrostatic pressure in both the proximal tubule and the peritubular capillaries.

Key words: Angiotensin II, angiotensin II antagonist, glomerulotubular balance, intratubular pressure, capillary pressure, rat nephron, proximal tubule

The flow rate of sodium chloride to the distal tubule is known to influence the renin activity and the hydrostatic pressure in the glomerular capillaries of a single rat nephron (29, 9, 10, 24, 32). In addition, infusion of Ileu⁸ angiotensin II [(Ileu⁸)-A II]—believed to be the circulating angiotensin II in the rat (21)—into 1st order peritubular capillaries (2) adjacent to the proximal tubule was found to elicit an increase in hydrostatic pressure in both the infused

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and the proximal tubule and a concurrent decrease in the rate of proximal fluid reabsorption (RPF_R) (28).

nts were designed to test the hypothesis that angiotensin II in its action on a of cells in vascular smooth muscle and the proximal tubule, acts homeostatically a constant hydrostatic pressure in the proximal tubule. Such effects will be f angiotensin II can be displaced from its receptor sites by a competitive antago- could produce a decrease in hydrostatic pressure in the peritubular capillaries renal tubule together with an increase in RPF/R. Accordingly peritubular f the rat kidney were infused with an analogue of angiotensin II, 1-sarcosine, ngiotensin II [(Sar¹ Ala⁷)-A II], which has been shown to act as a competitive n the action of angiotensin II on vascular smooth muscle in the rat (19, 26) and unctions cells in the rat adrenal cortex (26, 30).

Methods

We made on sodium pentobarbital anesthetized male Wistar rats, weighing between 230 species of the animals, surgical procedures, micropuncture and analytical techniques were described (17-28).

Plasma renin activity (PRA) was achieved by infusing isotonic saline at different rates. A rate of 2.0 ml min⁻¹ priming infusion sustained renal preparation of the kidney as completely followed by 1.2 ml min⁻¹ the maximum rate as 0.6 ml min⁻¹ without any priming. The maximum rate is saline containing 65 mCi mmole⁻¹ ¹⁴C-carboxylic acid (Radiochemical Centre, Amersham) used for most experiments involving tubular fluid collections, an intermediate rate, comprising infusions, sustained by 1.2 ml min⁻¹ throughout, was used in the remainder of these experiments associated with peritubular capillary infusion of (Sar¹, Ala⁸)-Ang II. The range in PRA of 0.1–1.0 ng ml⁻¹ h⁻¹ is achieved by the use of the maximum and the intermediate rates. This small range in infusion rates is unlikely to have exerted direct measurable influence on intratubular and extratubular processes.

measured in duplicate by radioimmunoassay (20) of blood collected from the femoral artery of the animal. In most of the experiments involving intratubular and capillary pressure measurements, samples for PRA determination were obtained a few minutes after making the pressure

Five solutions were made up freshly each day: one containing 20 $\mu\text{g ml}^{-1}$ (Dlu^3)-A II, the second 100 $\mu\text{g ml}^{-1}$ (Sar^1 Ala^8)-A II and third comprising 20 $\mu\text{g ml}^{-1}$ (Dlu^3)-A II plus (Sar^1 Ala^8)-A II. These solutions were prepared from aliquots of isotonic saline containing (Dlu^3)-A II (Schwartz Research, Orangeburg, N.Y. U.S.A.) and 0.01 $\mu\text{g ml}^{-1}$ (Sar^1 Ala^8)-A II (Pharmacia Co. Norwalk, N.Y. U.S.A.) maintained at 20°C in glass vials. A few drops of heparin were added to 1 to 3 ml of each solution for anticoagulation, this resulted in a final concentration of 10 $\mu\text{g ml}^{-1}$. Solutions were infused into the peritubular capillaries using the Sonenberg Desjardins perfusion system (28). At this infusion rate, 20 $\mu\text{g ml}^{-1}$ (Dlu^3)-A II represents a relatively high physiological dose (29).

micropipette (27-28) was used to measure hydrostatic pressure in 1st order peritubular capillaries with Ca^{2+} Ala^{2+} -A II and in proximal tubules associated with anastomosing infused capillaries. Additional measurements were made in nonanastomosing capillaries or in proximal tubules before use. To examine the ability of Ca^{2+} Ala^{2+} -A II to block the effect of (Dca^{2+}) -A II, these were repeated infusing the peritubular capillaries with (Dca^{2+}) -A II, or the mixture of (Dca^{2+}) -A II and Ca^{2+} Ala^{2+} -A II, or with isotonic saline alone.

of (Sar¹ Ala⁸)-A II on rat proximal nephron function was studied to determine whether the effect of angiotensin II on hydrostatic pressure in the proximal tubule is mediated wholly or in part by RPF. Peritubular capillaries were infused alternately with isotonic saline and either A II or the structure of (Ileu¹-A II plus (Sar¹ Ala⁸)-A II RPF. RPF, nephron GFR and hydrostatic pressure were measured in the adjacent tubules.

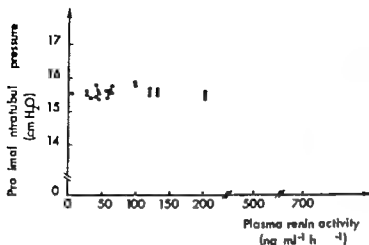


Fig. 1 Relationship between hydrostatic pressure in the proximal tubule and plasma renin activity (PRA). Variation in PRA achieved by using different rates of i.v. infusion of isotonic saline.

Timed quantitative fluid collections were made from the last superficial proximal segments. The tubular fluid collection time was approximately 130 s throughout. The mean ratio of the oil block insertion time to the tubular fluid collection time was between 0.09 and 0.10 for all sample groups. For all experiments, for fluid collections, the mean capillary infusion time was between 7 and 8 min. Kidney GFR and the femoral artery blood pressure were also recorded.

Nephron GFR was calculated from the tubular fluid to plasma isotope ratio, TF/P isotope, and the volume of tubular fluid collected per minute, V , using the following expression: $\text{nephron GFR} = \text{TF/P isotope} \times V$. The absolute rate of tubular fluid reabsorption to the site of puncture was calculated as follows: $\text{reabsorption GFR} = V - \text{nephron GFR}$. For statistical evaluation the two-tailed sign test was employed with a 5% level of significance (25).

Results

Hydrostatic pressure

a) *Intratubular pressure* (Fig. 1 and 2) Pressures in proximal tubules before infusion varied very little despite large differences in PRA from one rat to another.

Infusion of the peritubular capillaries with (Sar¹ Ala⁸)-A II caused a significant decrease in hydrostatic pressure in the adjacent proximal tubules ($p < 0.05$) (28 measurements in 11 rats). The magnitude of this decrease varied; the most striking fall in pressure occurred in rats with relatively high levels of PRA, suggesting that the angiotensin II antagonist inhibited endogenous angiotensin II.

The large variations in PRA in association with a constant intratubular pressure, coupled with the changes in hydrostatic pressure which could be induced by the infusion of (Sar¹ Ala⁸)-A II strongly suggest that angiotensin II is continuously exerting a regulatory effect on pressure in the proximal tubule.

With (Ileu⁸)-A II alone as the infusate, hydrostatic pressure in the proximal tubules was significantly increased ($p < 0.05$) (8 measurements in 6 rats), but no significant pressure change resulted from infusion of the (Ileu⁸)-A II plus (Sar¹ Ala⁸)-A II mixture (17 measurements in 10 rats).

b) *Capillary pressure* (Table I) Changes in capillary pressure were similar to those observed intratubularly. Infusion with (Sar¹ Ala⁸)-A II caused a significant decrease ($p < 0.05$) in peritubular capillary pressure; again the most striking reduction was observed in rats with relatively high PRA.

Fig. 1. Effect on hydrostatic pressure in the proximal tubule of peritubular capillary infusion (20 ml min⁻¹) with: 20 ng ml⁻¹ (Sar¹)₁-A II (squares); 333 ng ml⁻¹ (Ile¹ Ala²)₁-A II (triangles); 20 ng ml⁻¹ (Ileu¹)₁-A II (circles); 20 ng ml⁻¹ (Sar¹ Ala²)₁-A II mixture (circles) on total effective plasma renin activities (PRA). Mean Δ interstitial pressure are plotted as mean of PRA. Variation in PRA was achieved by varying rates of iv infusion of isotonic saline.

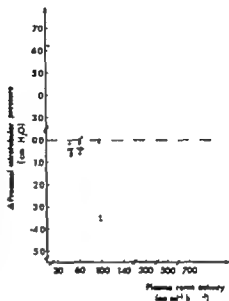


Table I. Effect on hydrostatic pressure in the peritubular capillaries of infusion (20 ml min⁻¹) with: (Ileu¹)₁-A II (20 ng ml⁻¹), (Sar¹ Ala²)₁-A II (333 ng ml⁻¹), (Ileu¹)₁-A II (20 ng ml⁻¹) plus (Sar¹ Ala²)₁-A II (333 ng ml⁻¹) mixture; isotonic saline. The mean value of each expt. is given. The number of measurements made and used to generate each mean value is given in parentheses.

Expt. no.	Capillary pressure measured (cmH ₂ O)	Capillary pressure inferred (cmH ₂ O)	Blood pressure (mmHg)	PRA (ng ml ⁻¹ h ⁻¹)
(Sar¹)₁-A II				
16.6 (1)	23.8 (1)	114	—	
16.0 (2)	21.5 (1)	130	—	
17.9 (2)	23.6 (7)	128	—	
17.4 (2)	22.7 (7)	126	62	
(Sar¹ Ala²)₁-A II				
13.4 (1)	12.4 (7)	126	119	
15.8 (2)	13.3 (2)	125	97	
16.0 (2)	13.4 (1)	116	45	
15.1 (7)	13.4 (1)	115	39	
17.5 (7)	15.9 (7)	116	57	
17.0 (7)	15.8 (2)	167	42	
17.3 (1)	16.2 (1)	120	66	
(Sar¹)₁-A II + (Sar¹ Ala²)₁-A II				
16.0 (1)	16.4 (1)	116	45	
15.2 (1)	15.0 (1)	114	39	
17.4 (2)	16.2 (2)	116	62	
Isotonic saline				
16.6 (1)	17.2 (2)	114	—	
15.9 (2)	16.7 (2)	117	—	

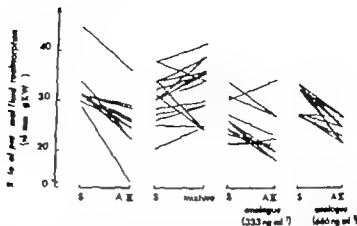


Fig. 3 Effect on the rate of proximal fluid reabsorption (RPFR) of peritubular capillary infusion (2 l/min) with 20 ng ml^{-1} (Ileu⁸)-A II 70 ng ml^{-1} (Ileu⁸)-A II plus 333 ng ml^{-1} (Sar¹ Ala⁸)-A II or 333 ng ml^{-1} (Sar¹ Ala⁸)-A II 666 ng ml^{-1} (Sar¹ Ala⁸)-A II. In each rat, tubular fluid collections made alternately from different tubules associated with peritubular capillary infusion of either isotonic saline (S) or one of the experimental solutions. The lines connect the individual values derived from a rat.

Infusion with (Ileu⁸)-A II produced an increase in capillary pressure, but no change resulted from infusion of the (Ileu⁸)-A II plus (Sar¹ Ala⁸)-A II mixture.

Isotonic saline infusion caused a small increase in capillary pressure, slightly greater than that previously reported for intratubular pressure (28).

Arterial blood pressure was not related to PRA level.

Proximal nephron function (Fig. 3, 4 and 5 values obtained previously with infusion (Ileu⁸)-A II are included for comparison)

Infusion with (Ileu⁸)-A II has been shown to cause a significant ($p < 0.05$) decrease in RPFR (28).

Infusion with the mixture of (Ileu⁸)-A II plus (Sar¹ Ala⁸)-A II failed to cause consistent changes in RPFR ($p > 0.05$), suggesting that the angiotensin II analogue blocked the observed effect of (Ileu⁸)-A II on RPFR.

No significant change in RPFR was caused by infusion with 333 ng ml^{-1} (Sar¹ Ala⁸)-A II but when the concentration was increased to 666 ng ml^{-1} , a decrease in RPFR was consistently observed.

Nephron GFR is unaffected by the infusion of (Ileu⁸)-A II (28). Infusion with 333 ng ml^{-1} (Sar¹ Ala⁸)-A II was similarly ineffective ($p > 0.05$), but nephron GFR increased slightly although not significantly ($p > 0.05$) when the mixture of (Ileu⁸)-A II plus (Sar¹ Ala⁸)-A II was infused. Infusion of 666 ng ml^{-1} (Sar¹ Ala⁸)-A II consistently resulted in a decrease in nephron GFR, despite a simultaneous substantial decrease in intratubular pressure.

Indices of whole kidney function and PRA level in experiments related to proximal nephron function

Mean kidney GFR and mean arterial blood pressure measured in association with infusion of each type of experimental solution or with infusion of isotonic saline are given in Table 1. Neither of the paired differences departed significantly from zero ($p > 0.05$).

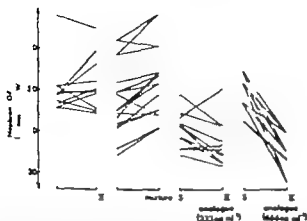


Fig. 4 Effect on nephron GFR of peritubular capillary infusion (20 ml min⁻¹) with: 20 ng ml⁻¹ (Ileu⁸)-A II, 20 ng ml⁻¹ (Ileu⁸)-A II plus 333 ng ml⁻¹ (Sar¹ Ala⁸)-A II mixture, 333 ng ml⁻¹ (Sar¹ Ala⁸)-A II, 666 ng ml⁻¹ (Sar¹ Ala⁸)-A II. In each rat, tubular fluid collections were made alternately from different sites associated with peritubular capillary infusion of either isotonic saline (S) or one of the experimental doses. The lines connect the individual values derived from each rat.

PRA was measured in 5 of the 9 expts. associated with (Ileu⁸)-A II infusion, 6 of the 7 with the mixture of (Ileu⁸)-A II plus (Sar¹ Ala⁸)-A II, 3 of the 6 with 333 ng ml⁻¹ (Sar¹ Ala⁸)-A II, and in both expts. with 666 ng ml⁻¹ (Sar¹ Ala⁸)-A II mean values for PRA (and \pm SE) respectively were 46 (5-83), 88 (60-130), 116 (64-202), and 158 (151-166) ng ml⁻¹ h⁻¹.

Discussion

The experimental data provide strong evidence that the hydrostatic pressure in the peritubular capillaries is maintained by the vascular action of angiotensin II and further indicate that angiotensin II plays a homeostatic role in the regulation of hydrostatic pressure in the proximal tubule. A constant flow rate to the distal tubule may result from stabilizing hydrostatic pressure in the proximal tubule.

The present results agree with earlier observations (5) that infusion of (Sar¹ Ala⁸)-A II into the renal artery of the sodium depleted dog decreased renal vascular resistance and increased renal blood flow. Infusion did not alter these functions in the normal dog. Renal sodium excretion did not change during (Sar¹ Ala⁸)-A II infusion. In the rabbit, similar observations have been made (18).

In some preliminary expts. (Steven, unpublished), alternate infusion of 20 ng ml⁻¹ and 80 ng ml⁻¹ (Ileu⁸)-A II into the same peritubular capillary resulted in a clearly visible reduction in capillary diameter in association with the more concentrated solution. The action of angiotensin II in stabilizing hydrostatic pressure in the capillaries could therefore be mediated partly by inducing changes in capillary diameter. This does not preclude the possibility that tubular results mainly from changes in postcapillary resistance.

Evaluation of the way in which angiotensin II stabilizes hydrostatic pressure in the proximal tubule on of the variables influencing this pressure, *i.e.* an



Fig. 5 Effect on hydrostatic pressure in the proximal tubule of peritubular capillary infusion (20 μ l) with 20 ng ml^{-1} (Ileu⁸)-A II 20 ng ml^{-1} (Ileu⁸)-A II plus 333 ng ml^{-1} (Sar¹ Ala⁹)-A II mixture 333 ng ml^{-1} (Sar¹ Ala⁹)-A II 666 ng ml^{-1} (Sar¹ Ala⁹)-A II. Measurements were made in each tubule before (control C) and during peritubular capillary infusion with one of the experimental solutions. The lines connect individual values derived from each rat. Results are given only from expts. involving tubular fluid collections.

analysis of nephron hydrodynamics. Since decreases in hydrostatic pressure along proximal and distal tubules are very small (7, 8, 15, 16, 31) there are effectively no resistances to fluid flow in each nephron: glomerular resistance, loop of Henle resistance and collecting duct resistance. For the total number of nephrons within the kidney each of these 3 resistances is connected in parallel. For analytical purposes, it is convenient to consider the "equivalent nephron" a hypothetical single nephron which has the same hydrodynamic characteristics as the entire population of nephrons in the kidney. For

TABLE II Indices of whole kidney function in the expts. related to proximal nephron function.

Infusate	GFR (ml min ⁻¹ gKW ⁻¹)		Mean arterial pressure (mmHg)	
	E ^a	S ^b	E ^a	S ^b
[Ileu ⁸]-A II 20 ng ml^{-1} (range)	1.37 (1.16-1.62)	1.51 (1.31-1.80)	118 (94-131)	118 (100-131)
[Ileu ⁸]-A II 20 ng ml^{-1} plus [Sar ¹ Ala ⁹]-A II 333 ng ml^{-1} (range)	1.47 (1.13-1.78)	1.52 (1.25-1.82)	111 (95-119)	112 (107-120)
[Sar ¹ Ala ⁹]-A II 333 ng ml^{-1} (range)	1.37 (1.14-1.60)	1.45 (1.28-1.65)	121 (108-124)	119 (107-126)
[Sar ¹ Ala ⁹]-A II 666 ng ml^{-1} (range)	1.22 (0.83-1.54)	1.33 (0.95-1.50)	129 (116-137)	133 (118-142)

Experimental infusate. ^b Isotonic saline infusate.

Neither of the paired differences departed significantly from zero (p > 0.05).

equivalent nephron, the pressure head, which may be termed the glomerular propulsive pressure (13), that drives fluid against the nephron resistance is equal to the difference between the mean glomerular capillary pressure, less the mean glomerular oncotic pressure, and the renal pelvic pressure.

Equivalent glomerular and loop of Henle resistances are found by dividing the respective resistances of the single nephron by the total number of nephrons in the kidney approximately 30 000 in the rat (11). Collecting duct resistance seems to be located mainly in the terminal collecting duct (17-22). Assuming that collecting duct resistance is exclusively terminal, an approximate value for equivalent collecting duct resistance is given by dividing the resistance of the single collecting duct by the number of terminal collecting ducts in the kidney approximately 20 in the rat (11).

These calculations involve some simplification, in that all nephrons are assumed to function in an identical manner but they provide an appropriate first approximation since recent measurements in the inner medulla of the rat kidney have shown that the hydrostatic pressure in the loop of Henle of the juxtamedullary nephron is similar to that of the superficial nephron (22). From experimental measurements in the hydropenic rat (1, 2, 3, 6, 7, 12, 14, 23-31), the following values may be derived: equivalent glomerular resistance 10^{-4} mmHg ml⁻¹ min, loop of Henle resistance $15 \cdot 10^{-4}$ mmHg ml⁻¹ min, collecting duct resistance $700 \cdot 10^{-4}$ mmHg ml⁻¹ min. The hydrodynamic resistance of the glomerular membrane is accordingly very small compared with tubular resistance, of which the great majority is encountered at the distal extreme of the tubular system.

Increasing the glomerular capillary pressure increases GFR and the urine flow rate, but the percentage of additional filtered fluid reabsorbed will be smaller than the percentage of fluid reabsorbed previously. This observation taken together with the distribution of hydrodynamic resistances calculated above will give the equivalent nephron the following definite characteristics, always assuming that tubular resistance is constant. GFR may be considerably changed by small alterations in RPFR but is influenced only to a small extent by changes in glomerular propulsive pressure. Conversely hydrostatic pressure in the proximal tubule will be relatively insensitive to changes in RPFR but will be sensitive to the most modest perturbations in glomerular propulsive pressure. Although constant tubular resistance has been assumed in the foregoing calculations, the inverse changes in tubular resistance which are known (17) to occur in response to variations in intratubular pressure will stabilize the hydrostatic pressure in the proximal tubule and further decrease the small effect of alterations in RPFR on this pressure.

Infusion of peritubular capillaries with 20 ng ml^{-1} (Ileu⁸)-A II resulted in a significant decrease in RPFR. Infusion with the mixture of 20 ng ml^{-1} (Ileu⁸)-A II plus 333 ng ml^{-1} (Sar¹-Ala⁹)-A II did not cause any consistent change in RPFR, which suggests that (Sar¹-Ala⁹)-A II blocked the effect of (Ileu⁸)-A II observed previously.

When 333 ng ml^{-1} (Sar¹-Ala⁹)-A II alone was similarly infused, no consistent change was found in RPFR despite the relatively high PRA levels. This finding is difficult to reconcile with the observation that (Sar¹-Ala⁹)-A II blocked both the vascular and tubular effects of (Ileu⁸)-A II. We can, as yet, offer no explanation for this discrepancy. Nevertheless, it follows from the foregoing outline of nephron hydrodynamic characteristics, that the influence of

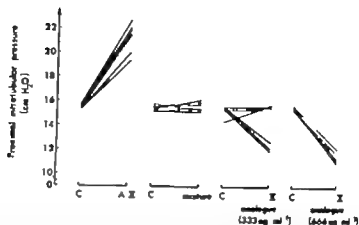


Fig. 5 Effect on hydrostatic pressure in the proximal tubule of peritubular capillary infusion (20 ml min⁻¹ with 20 ng ml⁻¹ (Ileu⁸)-A II 20 ng ml⁻¹ (Ileu⁸)-A II plus 333 ng ml⁻¹ (Sar¹ Ala⁹)-A II solvent: 333 ng ml⁻¹ (Sar¹ Ala⁹)-A II 666 ng ml⁻¹ (Sar¹ Ala⁹)-A II. Measurements were made in each tubule before (control C) and during peritubular capillary infusion with one of the experimental solutions. The lines connect the individual values derived from each rat. Results are given only from expts. involving tubular fluid collections.

analysis of nephron hydrodynamics. Since decreases in hydrostatic pressure along the proximal and distal tubules are very small (7, 8, 15, 16, 31), there are effectively only 3 resistances to fluid flow in each nephron: glomerular resistance, loop of Henle resistance, and collecting duct resistance. For the total number of nephrons within the kidney each of these 3 resistances is connected in parallel. For analytical purposes, it is convenient to consider the "equivalent nephron" a hypothetical single nephron which has the same hydrodynamic characteristics as the entire population of nephrons in the kidney. For the

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Experimental infusate. ^b Isotonic saline infusate.
Neither of the paired differences departed significantly from zero ($p > 0.0$)

angiotensin II or indeed of any substance, on proximal intratubular pressure cannot be effected by changes in RPF/R but must involve changes in either the glomerular pressure or the tubular resistance. The effect of angiotensin II on intratubular pressure is probably mediated by variations in the ultrafiltration pressure in the glomerular capillaries since it appears unlikely that the intratubular pressure could be regulated by changes in tubular resistance that is itself very sensitive to tubular pressure (P. Koch-Jensen and K. Steven, unpublished). An augmentation in glomerular capillary pressure increases tubular pressure and so increases the flow rate to the distal tubule. In addition, the increase in tubular pressure will result in a decrease in tubular resistance, causing a further increase in the flow rate to the distal tubule. An efficient regulatory mechanism for the glomerular capillary pressure will therefore stabilize the flow rate to the distal tubule and prevent over-elevation of the flow rate in relation to the reabsorptive capacity of the distal tubule and collecting duct.

Infusion of peritubular capillaries with 666 ng/ml (Sar¹-Ala⁸)-A II was performed in two experiments. In both a large decrease was observed in intratubular pressure, associated with a substantial decrease in nephron GFR and with a marked reduction in RPF/R. Despite the small number of observations, it is apparent that (Sar¹-Ala⁸)-A II in this concentration at the infusion rate employed, exerted an agonistic, angiotensin II-like effect on RPF/R while simultaneously antagonising the vascular action of angiotensin II.

The present expts. clearly indicate that angiotensin II is a physiological regulator of the hydrostatic pressure in the proximal tubule and the peritubular capillaries of the rat nephron. The effect of angiotensin II on intratubular pressure is probably mediated by variations in capillary pressure in the glomerular capillaries.

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***In Vivo* Turnover Rate of Acetylcholine in Rat Brain Parts at Elevated Steady-State Concentration of Plasma Choline**

By

SVEN-ÅKE ECKERNÄS, LENA SAHLSTRÖM and STEN-MAGNUS AQUILONIUS

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Abstract

ECKERNÄS, S. Å., L. SAHLSTRÖM and S. M. AQUILONIUS. *In vivo* turnover rate of acetylcholine in rat brain parts at elevated steady state concentration of plasma choline. Acta physiol. scand. 1977 101 404-410.

Cortical and striatal turnover rates of acetylcholine (TR_{ACh}) were estimated by applying steady-state tracer kinetics in rats killed by microwave irradiation following constant i.v. infusion of 3H -Ch. In controls TR_{ACh} was $3.6 \text{ nmol g}^{-1} \text{ min}^{-1}$ in the cortex and $23.8 \text{ nmol g}^{-1} \text{ min}^{-1}$ in the striatum. When steady state plasma concentrations of Ch were increased from 17 to $140 \mu\text{mol l}^{-1}$ by a 15-min infusion of 3H -labelled Ch the corresponding TR_{ACh} were $3.6 \text{ nmol g}^{-1} \text{ min}^{-1}$ and $14 \text{ nmol g}^{-1} \text{ min}^{-1}$ respectively. These results indicate that increased plasma level of Ch is not accompanied by increased synthesis of brain ACh.

Key words. Acetylcholine, turnover rate, plasma choline, rat brain parts.

Several regulatory mechanisms for the synthesis of acetylcholine (ACh) in the cholinergic neurons have been suggested (for ref. see Browning 1976, Baker 1976, Haubrich 1976). It seems probable that the availability of choline (Ch) in the nerve terminal is the rate-limiting step in ACh synthesis (Eisenstadt and Schwartz 1973, Simon *et al.* 1976). Recently it has been suggested by several investigators that an increase in plasma Ch concentration can increase the availability of the ACh precursor and thus enhance the rate of ACh synthesis. This suggestion is based on the observation that brain ACh levels are elevated by the systemic administration of a large dose of Ch (Cohen and Wurtman 1975, 1976, Haubrich *et al.* 1974, 1975, Wurtman and Fernstrom 1976). However, a direct agonistic effect of Ch cannot be ruled out, since no attempts have been made to determine the brain turnover rate of ACh at an increased plasma Ch level.

In the present paper we have applied the principles of steady state tracer kinetics (Zihner *et al.* 1960) following a constant i.v. infusion of tritiated Ch (3H -Ch) for the calculation of brain ACh turnover rates in rats with different steady state concentrations of Ch in plasma.

Methods

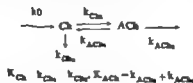
General Male Sprague-Dawley albino rats weighing 220-240 g were used. The animals were housed for one week with food *ad libitum* before the experiments, which took place at the same time of the day. The rats were killed by focusing beams of microwave on the head for 17 (5 kW 2 450 MHz, Magnetostat, Gerling Weiss Inc., USA). Brain temperature measured 5 mm below the bregma 10 after irradiation is about 35°C. These conditions are known to stop poor marker changes in brain Ch and ACh (Schmidt 1976, Sanoaka *et al.* 1973, Nordberg 1977). Immediately after sacrifice 100 µl of blood as drawn by means of heart puncture and the paraventricular and striata were dissected out. The tissue samples were homogenized (1 ml ice-cold 0.1 M formic acid in acetone) and further processed as described earlier (Eckardt and Aquilonius 1977).

Determination of Ch and ACh The brain Ch and ACh concentrations and plasma Ch concentration were analysed by radio-chemical procedure (Eckardt and Aquilonius 1977 a, b).

Measurement of radioactive Ch and ACh 1 µl aliquots of the tissue extracts ACh and Ch are separated by means of high voltage paper electrophoresis (Aquilonius and Eckardt 1976). After localization with iodine vapour the spots are cut out and eluted in 4 ml of methanol in scintillation vial. After adding 10 ml of scintillation cocktail (Perambled II Packard 77 mg ml⁻¹ toluene), the vials were kept in darkness for 12 h before the radioactivity is measured by liquid scintillation counting (Liscomp-300, Scintec Nuclear Chicago Division).

Estimation of ACh turnover rate A tracer dose (47 nmol kg⁻¹ min⁻¹) of H-Ch (10.6 Ci mmol⁻¹ Amersham, England) was infused (1 ml kg⁻¹ min⁻¹ 0.5 µl Ci kg⁻¹ min⁻¹) for 2 or 3 min, using a syringe. During the infusion the animals were in place in the holder, then the microwave exposure and the rats were killed immediately after the infusion.

In some experiments the steady-state concentrations of plasma Ch was increased by means of 15-min infusion of unlabeled Ch (0.25 ml kg⁻¹ min⁻¹ 15 µmol kg⁻¹ min⁻¹) before the infusion of H-Ch. The two infusions were then continued simultaneously for further 2 or 3 min. Brain ACh turnover rates are estimated by measuring the change with time of the specific radioactivity of brain Ch (S_{Ch}) and ACh (S_{ACh}). The following kinetic model was assumed:



K_{Ch} and K_{ACh} denote the fractional rate constants of brain Ch and ACh compartments, respectively, i.e. the fractions of the compartments that are renewed per unit of time (min⁻¹). The turnover rate of ACh (TR_{ACh} nmol g⁻¹ min⁻¹) is then calculated from the following formula:

$$TR_{ACh} = K_{ACh} (\text{ACh concentration}) \quad (1)$$

Under the assumption that steady-state conditions exist and that the change in brain S_{Ch} and S_{ACh} with time can be mathematically handled as if brain Ch and ACh are each in a homogeneous compartment, here random processes prevail, the following equation describes the change of S_{ACh} with time (Zahradnik 1967):

$$\frac{dS_{ACh}}{dt} = K_{ACh}(S_{Ch} - S_{ACh}) \quad (2)$$

The equation can be solved for K_{ACh} as follows. The function $f(t)$ is assumed to be any function which describes the change of S_{Ch} with time ($S_{Ch} = f(t)$). $f(t)$ is inserted in equation 2 to yield:

$$\frac{dS_{ACh}}{dt} = K_{ACh}(f(t) - S_{ACh}) \quad (3)$$

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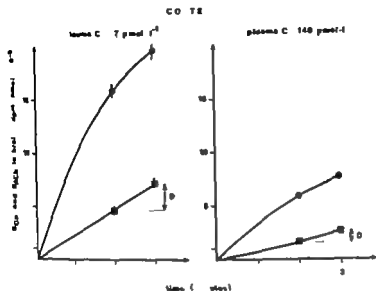
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Cortical and striatal turnover rates of acetylcholine TR_{ACh} were estimated by applying steady-state tracer kinetics in rats killed by microwave irradiation following a constant i.v. infusion of H-Ch. In control rats TR_{ACh} was $3.6 \text{ nmol g}^{-1} \text{ min}^{-1}$ in the cortex and $23.8 \text{ nmol g}^{-1} \text{ min}^{-1}$ in the striatum. When steady state plasma concentrations of Ch were increased from 17 to $140 \text{ } \mu\text{mol l}^{-1}$ by 15-min infusion of ^3H -labelled Ch the corresponding TR_{ACh} were $3.6 \text{ nmol g}^{-1} \text{ min}^{-1}$ and $21.4 \text{ nmol g}^{-1} \text{ min}^{-1}$ respectively. These results indicate that increased plasma levels of Ch are not accompanied by increased synthesis of brain ACh.

Key words: Acetylcholine, turnover rate, plasma choline, rat brain parts

Several regulatory mechanisms for the synthesis of acetylcholine (ACh) in the cholinergic neurons have been suggested (for ref. see Browning 1976, Baker 1976, Haubrich 1976). It seems probable that the availability of choline (Ch) in the nerve terminal is the rate-limiting step in ACh synthesis (Eisenstadt and Schwartz 1975, Simon *et al.* 1976). Recently it has been suggested by several investigators that an increase in plasma Ch concentration can increase the availability of the ACh precursor and thus enhance the rate of ACh synthesis. This suggestion is based on the observation that brain ACh levels are elevated by the systemic administration of a large dose of Ch (Cohen and Wurtman 1975, 1976, Haubrich *et al.* 1974, 1975, Wurtman and Fernstrom 1976). However, a direct agonistic effect of Ch cannot be ruled out, since no attempts have been made to determine the brain turnover rate of ACh at an increased plasma Ch level.

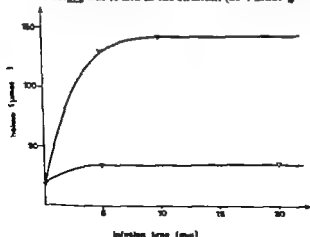
In the present paper we have applied the principles of steady-state tracer kinetics (Zihr *et al.* 1960) following a constant i.v. infusion of tritiated Ch (H-Ch) for the calculation of brain ACh turnover rates in rats with different steady state concentrations of Ch in plasma.



Specific radioactivity of Ch and ACh in the cortex during constant infusion of ^3H -Ch at rates infused into vein alone or with infused Ch. \circ — \circ S_{Ch} \bullet — \bullet S_{ACh} $K_{\text{ACh}} = D/(\text{shaded area})$.

the ACh concentration was slightly increased (84 nmol g^{-1}), while the cortical seemed to be unchanged.

Fig. 1 and 2 shows the alteration in S_{ACh} and S_{Ch} in the brain during a constant infusion of ^3H -Ch at different steady-state levels of plasma Ch. The calculated TR_{Ch} of saline treated animals was $23.8 \text{ g}^{-1} \text{ min}^{-1}$ in the striatum and $3.6 \text{ nmol g}^{-1} \text{ min}^{-1}$ in the cortex (Table I). Elevation of plasma Ch levels seemed to have no effect on cortical TR_{Ch} ($3.6 \text{ nmol g}^{-1} \text{ min}^{-1}$), a slight decrease in TR_{ACh} was found in the striatum ($21.4 \text{ nmol g}^{-1} \text{ min}^{-1}$ Table I).



The change in plasma Ch concentration in carotid blood of anesthetized rats during constant infusion of unlabeled Ch. ∇ — ∇ $15 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ ∇ — ∇ $2.5 \mu\text{mol kg}^{-1} \text{ min}^{-1}$

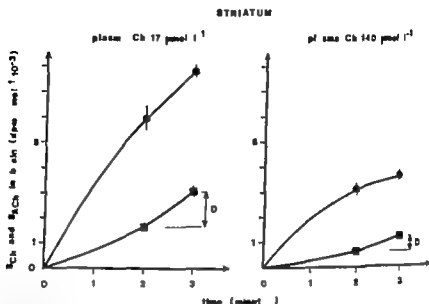


Fig. 1 Specific radioactivity of Ch and ACh in the striatum during an i.v. infusion of ^3H -Ch in rats infused for 15 min with saline or unlabelled Ch. \circ — \circ S_{Ch} \square — \square S_{ACh} $K_{\text{ACh}} = D$ (shaded area).

which can be integrated between two time points t_1 and t_2 .

$$S_{\text{ACh}_2} - S_{\text{ACh}_1} = K_{\text{ACh}} \left(\int_{t_1}^{t_2} f(t) dt - \int_{t_1}^{t_2} S_{\text{ACh}} dt \right)$$

The difference at the left is equal to D and the quantity between the square brackets is equal to the shaded area in Fig. 1 and 2. Thus

$$K_{\text{ACh}} = \frac{D}{\text{shaded area}}$$

By approximating the changes in S_{Ch} and S_{ACh} between 2 and 3 min to straight lines, K_{ACh} can easily be calculated.

In some rats the brain blood volume was estimated with ^3H -dextran (Aquilonus and Eckernäs 1976) and it was concluded that Ch treatment did not alter the remaining blood volume in brain tissue.

Results

Elevation of plasma Ch. In Fig. 3 the change in plasma Ch concentration during a constant i.v. infusion of unlabelled Ch ($15 \mu\text{mol kg}^{-1} \text{ min}^{-1}$ and $2.5 \mu\text{mol kg}^{-1} \text{ min}^{-1}$) to anesthetized rats is shown. The steady state level is reached within 10 min (Fig. 3) and seems to be linearly related to the infusion rate (Fig. 4). In the unanesthetized rats, used in the turnover calculations, the final steady-state level of plasma Ch following the infusion of unlabelled Ch was also about $140 \mu\text{mol l}^{-1}$.

Brain Ch and ACh concentrations. The results are presented in Table 1. In rats infused with saline the striatal Ch and ACh concentrations were 33 ± 1.2 and $76 \pm 2.9 \text{ nmol g}^{-1}$ respectively while the corresponding values in the cortex were 23 ± 2.5 and $16 \pm 1.0 \text{ nmol g}^{-1}$.

When plasma levels of Ch were increased to $140 \mu\text{mol l}^{-1}$ by means of a constant i.v. infusion of unlabelled Ch for 15 min, significantly increased concentrations of brain Ch were detected both in the striatum (53 nmol g^{-1}) and in the cortex (59 nmol g^{-1}). In the

was calculated from D (shaded area) in Fig. 1 and 2. The theoretical error introduced by this simplification is negligible in consideration of the experimental error inherent in each value (Fig. 1 and 2). The estimated TR_{ACh} values in control rats are almost identical with those recently reported by Racine et al. (1974).

Our problem with the estimation of TR_{Ch} following administration of Ch is that the system must be at steady-state if the equations described under Methods shall be valid. To fulfil this criterion the plasma Ch concentrations had to be elevated by means of a constant infusion of radiolabelled Ch prior to an infusion of H-Ch. As shown in Fig. 3 steady-state levels of plasma Ch are rapidly achieved during an i.v. infusion and these levels seemed to be directly related to the infusion rate (Fig. 4), which is in agreement with the result of Free et al. (1975). Thus, after a 15-min infusion of unlabelled Ch, the plasma Ch must fulfil the steady-state criterion. From Fig. 4 it is obvious that the infusion of H-Ch (47 nmol min^{-1}) does not significantly perturb the steady-state conditions of Ch.

The most elevated plasma Ch level ($\sim 140 \text{ } \mu\text{mol l}^{-1}$) is of the same magnitude as the peak values shown to be paralleled by increased brain ACh concentrations in the studies by Haubrich et al. (1975) using i.p. administration of Ch. In the present study the Ch concentrations were significantly increased both in the cortex and in the striatum following the infusion of Ch, while ACh was increased slightly (11%) in the striatum only (Table I). This is in accordance with the idea that an increased concentration of plasma Ch will be paralleled by increased steady-state concentrations of brain Ch and ACh (Cohen and Wurt 1973, Haubrich et al. 1974, 1975). However, in rats with plasma Ch concentrations elevated to $140 \text{ } \mu\text{mol l}^{-1}$ no definite changes in the regional ACh turnover rates were observed. These findings thus contradict the suggestion that administration of Ch will enhance neuronal synthesis of ACh in the brain.

It is well known that centrally acting muscarinic agonists (e.g. oxotremorine) increase the ACh concentration in the brain but decrease TR_{Ch} (Nordberg 1977, Trabocchi et al. 1975). Thus, at present the most probable explanation for the slight but significant increase in striatal ACh concentration and the minute reductions of striatal TR_{ACh} would seem to be a direct agonistic effect of Ch itself in some receptor-close compartment, resulting in feedback inhibition of neuronal activity or that the increase in ACh concentration is localized at a non-releasable pool of ACh.

However, the present results do not necessarily rule out the possibility that cholinergic function will be enhanced by the administration of Ch under pathological conditions (Aquilante and Eckstein 1975, 1977).

We are indebted to Mrs Inger Falk and Miss Lisbeth Lehtipalo for technical assistance. Our thanks are due to Professor B. Holmstedt for kindly allowing us to use the microanalytical equipment at the Department of Toxicology at Karolinska Institute, Stockholm.

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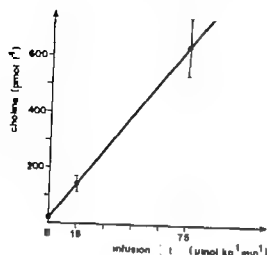


Fig. 4. Steady-state levels of plasma Ch as a function of the rate of infusion of unlabeled Ch.

Discussion

The aim of this study was to evaluate the proposals recently put forward by several authors (Cohen and Wurtman 1975, 1976, Haubrich *et al.* 1974, 1975, Wurtman and Fernstrom 1976) that increased concentrations of plasma Ch stimulate the synthesis of brain ACh.

Under steady state conditions an enhanced transmitter synthesis must be balanced by an increased transmitter outflow and thus result in an increased turnover rate of the transmitter (Zilversmit 1960).

The validity of the equations formulated by Zilversmit and co-workers (for review see Zilversmit 1960) for calculation of the brain ACh turnover rate would seem questionable since the nature and number of various "pools" of Ch (Aquilionius and Eckernäs 1976) and ACh (Jenden *et al.* 1974, Nordberg 1977) are unknown components. It is also known at least *in vitro* that newly synthesized ACh is preferentially released (Collier 1969, Potkin 1970, Molenaar and Polak 1976). However, it has been clearly shown by Cheney *et al.* (1975) that these equations do seem to be valid for the calculation of ACh turnover rate, at least during the first 20 min, following the administration of radioactive precursor.

In the present study a tracer dose of ^3H -Ch was infused i.v. in rats and the S_{Ch} and S_{ACh} of brain tissue were measured after a 2 and 3 min infusion. The change of brain S_{Ch} and S_{ACh} with time was approximated to straight lines in the time interval $\Delta t = 3$ min and K_{ACh}

TABLE I. Cholinergic parameters in brain regions of the rat at different steady-state concentrations of plasma Ch.

Brain part and steady-state concentration of plasma Ch	Ch (nmol g ⁻¹) $\bar{x} \pm \text{S.E.}$	ACh (nmol g ⁻¹) $\bar{x} \pm \text{S.E.}$	Fractional rat constant K_{ACh} (min ⁻¹)	Turnover rate TR_{ACh} (mol g ⁻¹ min ⁻¹)
Cortex (plasma Ch 17 $\mu\text{mol l}^{-1}$ n=18)	23 ± 2.4	16 ± 1.6	0.22	3.6
Cortex (plasma Ch 140 $\mu\text{mol l}^{-1}$ n=15)	59 ± 3.7^a	14 ± 1.7	0.26	3.6
Striatum (plasma Ch 17 $\mu\text{mol l}^{-1}$ n=23)	33 ± 1.22	76 ± 4.9	0.31	23.8
Striatum (plasma Ch 140 $\mu\text{mol l}^{-1}$ n=22)	53 ± 4.0^a	84 ± 3.0	0.26	21.4

^a $p < 0.02$, ^b $p < 0.001$ (Student's *t* test).

was calculated from D_1 (shaded area) in Fig. 1 and 2. The theoretical error introduced by this simplification is negligible in consideration of the experimental error inherent in each value (Fig. 1 and 2). The estimated TR_{ACh} values in control rats are almost identical with those recently reported by Racagni *et al.* (1974).

One problem with the estimation of TR_{ACh} following administration of Ch is that the system must be at steady-state if the equations described under Methods shall be valid. To fulfil this criterion the plasma Ch concentrations had to be elevated by means of a constant Lv infusion of unlabelled Ch prior to an infusion of 3H -Ch. As shown in Fig. 3, steady-state levels of plasma Ch are rapidly achieved during an Lv infusion and these levels seemed to be linearly related to the infusion rate (Fig. 4), which is in agreement with the result of Freeman *et al.* (1975). Thus, after a 15-min infusion of unlabelled Ch, the plasma Ch must fulfil the steady-state criterion. From Fig. 4 it is obvious that the infusion of 3H -Ch ($47 \text{ nmol l}^{-1} \text{ min}^{-1}$) does not significantly perturb the steady-state conditions of Ch.

The mean elevated plasma Ch level ($\sim 140 \mu\text{mol l}^{-1}$) is of the same magnitude as the peak values shown to be paralleled by increased brain ACh concentrations in the studies by Hämboch *et al.* (1975) using Ip. administration of Ch. In the present study the Ch concentrations were significantly increased both in the cortex and in the striatum following the infusion of Ch, while ACh was increased slightly (11%) in the striatum only (Table I). This is in accordance with the idea that an increased concentration of plasma Ch will be paralleled by increased steady-state concentrations of brain Ch and ACh (Cohen and Wurtman 1975; Hämboch *et al.* 1974, 1975). However, in rats with plasma Ch concentrations elevated to $140 \mu\text{mol l}^{-1}$ no definite changes in the regional ACh turnover rates were observed. These findings thus contradict the suggestion that administration of Ch will enhance neuronal synthesis of ACh in the brain.

It is well known that centrally active muscarinic agonists (e.g. oxotremorine) increase the 3H -ACh concentration in the brain but decrease TR_{ACh} (Nordberg 1977; Trabucchi *et al.* 1975). Thus, at present the most probable explanation for the slight but significant increase in striatal ACh concentration and the minute reductions of striatal TR_{ACh} would seem to be a direct agonistic effect of Ch itself in some receptor-close compartment, resulting in feedback inhibition of neuronal activity or that the increase in ACh concentration is localized to the "non-releasable pool" of ACh.

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Effect of the Preceding Day's Energy Intake on the Total Energy Cost of Light Exercise

By

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Abstract

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The effect of variation in the preceding day's dietary energy intake—4, 10 and 18 MJ—on the oxygen consumption and respiratory quotient at rest and at work before and after a test meal (4 MJ) was investigated. 7 normal male subjects were studied at 3 different experimental situations. During the first study the subjects had an energy intake of 10 MJ and normal activity. The following morning measurements of \dot{V}_O_2 and \dot{V}_{O_2}/\dot{V}_E were performed at rest and at work on a bicycle ergometer (24 W) before and after the test meal. The same measurements were performed on the following day but the dietary intake between the two sets of measurements was then either 4, 6 or 14 MJ. No significant effect was found of the preceding day's dietary energy intake on the oxygen consumption or on the energy transformed during rest, during exercise before and after the test meal. The results do not support the conclusions of previously published studies, proposing significant feedback mechanisms tending to control the body energy content by regulation of the energy expenditure through chemical or mechanical uncouplers.

The ways in which human beings control their energy balance are poorly understood. It has been suggested that control takes place by regulation of both energy intake and energy expenditure, but neither the relative importance nor the mechanisms of these processes have been adequately defined. The literature up to 1973 has been reviewed by Garrow (1974), see also Sims (1976).

Müller-Mimford and Stock (1967) and Apfelbaum, Bontsaron and Lacates (1971) have published results that can be interpreted in terms of changes in efficiency during work when energy intake has been markedly changed for several weeks. Recently Müller and Mac (1975) have presented results, which indicate that the cost of light arm exercise varies with the energy content, at normal composition, of the preceding day's food. This finding suggests the existence of a feedback system operating on the efficiency of transformation of chemical energy also after a short disturbance in energy intake. The data of Müller and Mac do not allow a definite interpretation of the quantitative role of the suggested mechanism, but indicate that a significant part of the surplus energy intake on one day

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By

LARS GABBY and OLE LAMBERT

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Abstract

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can be dissipated during the next day and that quite a considerable part of an acute deficit can be recovered by increased efficiency of utilization of the next day's food intake. If substantiated and found to be valid for other activities as well, these findings have far-reaching implications for our understanding of how the energy balance is regulated.

The present work was undertaken in order to obtain additional information on the significance of the phenomenon described by Miller and Wise. We have measured the oxygen consumption and carbon dioxide elimination during light bicycle exercise (36 W) before and after a test meal on different days preceded by a day in which the food energy intake was varied between 4 and 18 MJ. The results show that under these conditions the variation of the total cost of this type of exercise with the preceding day's energy intake is either absent or so small as to be of questionable significance in the control system.

Materials and methods

The observations were performed on 7 apparently healthy male subjects (average age = 59 ± 3 years, weight = 78.0 ± 6.9 kg, height = 183 ± 5.6 cm).

Experimental design. This is illustrated in Fig. 1 and was as follows. On the first day of study the subjects performed their ordinary work and other activities with the exception that all their meals were prepared in the laboratory according to a standard procedure and with a fixed composition and amount so that it contained 10 MJ in total (see below). On the second day of study the subjects came to the laboratory in the morning after having fasted for 12–14 h. They rested under standard conditions for 15 min whereupon the resting oxygen consumption and carbon dioxide elimination were measured during 15 min. Immediately thereafter the subjects performed 36 W for 30 min on a bicycle ergometer (see below) and the respiratory gas exchange was measured during the last 15 min of the period. A test meal containing 4 MJ (see below) was then given and the subjects rested horizontally for 30 min after which the same work and measurements were performed as before the test meal. The remaining part of the day was spent with ordinary activities but the food taken was restricted to that prepared in the laboratory so that an additional energy intake with a fixed composition of 0, 6 or 14 MJ was ensured. On the third day of the experiment, the schedule of day 2 was repeated with the exception that the food intake after the measurements was free. Thus, measurements of oxygen consumption and carbon dioxide elimination were performed on each subject at rest, during exercise before the test meal and during exercise after the test meal on 3 different occasions, each consisting of 2 consecutive days of which the first followed a day with an intake of 10 MJ and the second followed a day with an energy intake of 4, 10 or 18 MJ.

Work performance and respiratory gas exchange

The oxygen consumption and carbon dioxide elimination were measured using a low-resistance modified Otis-McKerrow valve and the Douglas bag method. The bags were preinflated by the subjects immediately before the actual collection of expired air in order to ensure that the dead space in the bags had a known and oxygen and carbon dioxide concentrations close to those resulting from the actual measurements. Three bags were collected for each experimental period. The volume was measured in a calibrated 10-l spirometer and the oxygen and carbon dioxide concentrations were measured with a Servomex OA 14A oxygen analyser and a Beckman LBI carbon dioxide analyser respectively. Both instruments were calibrated using several gas mixtures with known oxygen and carbon dioxide concentrations measured by the micro-Scholander technique (P. F. Scholander 1967). The expiratory air was dried in a silica gel filter before admitted to the analysers.

From every third Douglas bag, 5 ml samples were drawn and the concentrations of oxygen and carbon dioxide were also measured using the micro-Scholander technique. This procedure was performed in order to check the stability of the instruments used for gas analysis. The mean value and the standard deviation of the 64 differences (instrument vs. micro-Scholander) in oxygen concentration were $0.03 \pm 0.05\%$ (absolute) and the corresponding values for carbon dioxide concentration were $0.01–0.05\%$ (absolute).

The Monark bicycle ergometer was calibrated before and several times during the test period.

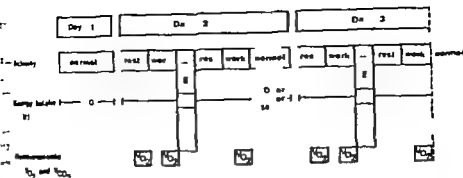


Diagram showing the experimental design

Fig. 1 The experimental design repeated once a week over 3 work periods. Day 1 The subjects ate all the meals at the laboratory (10 MJ), no measurements are performed. Day 2 and 3 V_{O_2} and V_{CO_2} were measured at the end of the rest period and work period before the test meal (4 MJ), and at the end of the work period after the meal. Between these two days measurements at meals are taken at the laboratory. The energy contained in these meals were either 0-6 or 14 MJ.

according to the manufacturer's manual. The ordinary pendulum was replaced by smaller pendulums in order to achieve greater sensitivity at the given work load. The subjects worked with 60 revolutions per minute as controlled by metronomes and the work intensity was 36 W. Measurements of heart rate and respiratory frequency were performed both during rest and exercise. The heart rate was measured by ECG registrations with digital display in combination with an ink-writer. The respiratory frequency was measured with a thermocouple transducer placed in the valve close to the mouthpiece.

In 6 of the subjects, all expts. are performed with one test interval on the same days in order to obtain similar patterns of daily physical activity. In one subject, all expts. were performed consecutively. The results from this subject did not differ from those found in the other 5 subjects. All the subjects followed the same dietary schedule: fast the 10-10 MJ expt. then the 10-4 MJ and finally the 10-18 MJ expt.

Composition and energy content of food

Among the food items commonly used in Denmark, suitable number was selected which could easily be weighed and weighed 3 standard meals—breakfast, lunch and dinner—were prepared by commonly used cooking methods with the exception that no rice was fried with fat or oil. Each test was weighed and the composition and energy content of each meal were calculated according to Helms (1973). The daily intake of 18 MJ was achieved by increasing the amount of each item in lunch and dinner by a factor of 2.5.

The energy content of one day's meals, from each of the 4, 10 and 18 MJ days, was decreased by

Table 1 Composition and energy content of meals

Meal	Composition, of total energy as				Energy content, MJ	
	Carbohydrate	Fat	Protein	Alcohol	Calculated	Measured
Breakfast (10 MJ)	47.8	36.7	14.8	0	4.2	4.24
Lunch	35.2	40.7	17.7	7.0	2.9	
Dinner	42.6	37.3	14.1	6.0	3.4	
Total 10 MJ	42.6	37.9	15.3	1.9	10.5	10.2
Total 18 MJ	41.1	38.3	15.3	5.0	18.2	17.2
Total 4 MJ	47.8	36.7	14.8	0	4.2	4.24

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Materials and methods

The observations were performed on 7 apparently healthy male subjects (average age = 25.9 ± 3 years, weight = 78.0 ± 6.9 kg, height = 182 ± 5.6 cm).

Experimental design This is illustrated in Fig. 1 and was as follows. On the first day of study the subjects performed their ordinary work and other activities with the exception that all their meals were prepared in the laboratory according to a standard procedure and with a fixed composition and amount so that it contained 10 MJ in total (see below). On the second day of study the subjects came to the laboratory in the morning after having fasted for 12–14 h. They rested under standard conditions for 30 min whereupon the resting oxygen consumption and carbon dioxide elimination were measured during 15 min. Immediately thereafter the subjects performed 36 W for 30 min on a bicycle ergometer (see below) and the respiratory gas exchange was measured during the last 15 min of the period. A test meal containing 4 MJ (see below) was then given and the subjects rested horizontally for 30 min after which the same work and measurements were performed as before the test meal. The remaining part of the day was spent with ordinary activities but the food taken was restricted to that prepared in the laboratory so that the additional energy intake with a fixed composition of 0, 6 or 14 MJ was ensured. On the third day of study, i.e., the schedule of day 2 was repeated with the exception that the food intake after the measurements was free. Thus, measurements of oxygen consumption and carbon dioxide elimination were performed on each subject at rest, during exercise before the test meal and during exercise after the test meal on different occasions, each consisting of 2 consecutive days of which the first followed a day with an energy intake of 10 MJ and the second followed a day with an energy intake of 4, 10 or 18 MJ.

Work performance and respiratory gas exchange

The oxygen consumption and carbon dioxide elimination were measured using a low-resistance modification of the Otis McKerrow valve and the Douglas bag method. The bags were preinflated by the subjects immediately before the actual collection of expired air in order to ensure that the dead space in the bags had a composition of oxygen and carbon dioxide concentrations close to those resulting from the actual measurements. Three bags were collected for each experimental period. The volume was measured in a calibrated 150-l spirometer and the oxygen and carbon dioxide concentrations were measured with Servomex OA 1 oxygen analyser and Beckman LBI carbon dioxide analyser respectively. Both instruments were calibrated using several gas mixtures with oxygen and carbon dioxide concentrations measured by the micro-Scholander technique (P. F. Scholander 1967). The expiratory air was dried in a silicagel filter before being admitted to the analysers.

From every third Douglas bag, 5 ml samples were drawn and the concentrations of oxygen and carbon dioxide were also measured using the micro-Scholander technique. This procedure was performed in order to check the stability of the instruments used for gas analysis. The mean value and the standard deviation of the 64 differences (instrument vs. micro-Scholander) in oxygen concentration were $0.01 \pm 0.03\%$ (absolute) and the corresponding values for carbon dioxide concentration were $+0.03 \pm 0.05\%$ (absolute).

The Monark bicycle ergometer was calibrated before and several times during the test period.

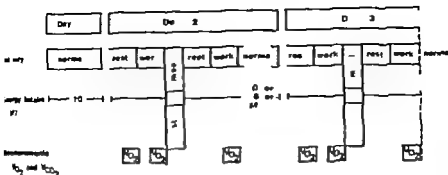


Diagram showing experimental design

Fig. 1 The experimental design repeated once a week over 3 weeks period. Day 1 The subjects ate all the meals at the laboratory (10 MJ), no measurements are performed. Day 2 and 3 V_{O_2} and V_{CO_2} were measured at the end of the rest period and work period before the test meal (4 MJ), and at the end of the work period after the meal. Between these two days measurements are taken at the laboratory. The energy contained in these meals were either 0-6 or 14 MJ.

working in the manufacturer's manual. The ordinary pedalism was replaced by smaller pedalism (25 W) in order to achieve greater sensitivity at the given work load. The subjects worked at 60 revolutions per minute as indicated by a metronome and the work intensity was 36 W. Measurements of heart rate and respiratory frequency were performed both during rest and exercise. The heart rate was measured by ECG registration with digital display in combination with an ink-writer. The respiratory frequency was measured with a thermoelectric transducer placed in the valve close to the mouthpiece.

In 6 of the subjects, all experiments were performed with one week intervals on the same days in order to obtain similar patterns of daily physical activity. In one subject, all experiments were performed consecutively. The meals from this subject did not differ from those found in the other 5 subjects. All the subjects followed the same dietary schedule: first the 10-10 MJ experiment, then the 10-6 MJ and finally the 10-18 MJ experiment.

Composition and energy content of food

As the food eaten commonly used in Denmark, suitable number was selected which could easily be divided and weighed. 3 standard meals—breakfast, lunch and dinner—were prepared by commonly used cooking methods, with the exception that no steam was fried with fat or oil. Each meal was weighed and the composition and energy content of each meal were calculated according to Helander (1973). The daily intake of 18 MJ was achieved by increasing the amount of each meal in lunch and dinner by a factor of 2.25.

The energy content of one day's meals, from each of the 4, 10 and 18 MJ days, was determined by

Table 1 Composition and energy content of meals

Meal	Composition, of total energy as				Energy content, MJ	
	Carbohydrate	Fat	Protein	Alcohol	Calculated	Measured
Breakfast (one meal)	47.8	36.7	14.8	0	4.2	4.24
Lunch	39.2	40.7	17.7	7.0	2.9	
Dinner	42.6	37.3	14.1	6.0	3.4	
Total 10 MJ	42.6	37.9	15.3	3.9	10.5	10.2
Total 18 MJ	41.1	38.3	15.5	5.0	18.2	17.2
Total 4 MJ	47.8	36.7	14.8	0	4.2	4.24

can be dissipated during the next day and that quite a considerable part of an deficit can be recovered by increased efficiency of utilization of the next day's food. If substantiated and found to be valid for other activities as well, these findings have reaching implications for our understanding of how the energy balance is regulated.

The present work was undertaken in order to obtain additional information on the significance of the phenomenon described by Miller and Wise. We have measured oxygen consumption and carbon dioxide elimination during light bicycle exercise before and after a test meal on different days preceded by a day in which the food intake was varied between 4 and 18 MJ. The results show that under these conditions the variation of the total cost of this type of exercise with the preceding day's energy intake is either absent or so small as to be of questionable significance in the control system.

Materials and methods

The observations were performed on 7 apparently healthy male subjects (average age 25.9 years, weight = 70 ± 6.9 kg, height = 182 ± 5.6 cm).

Experimental design. This is illustrated in Fig. 1 and was as follows. On the first day the subjects performed their ordinary work and other activities with the exception that all their meals were prepared in the laboratory according to a standard procedure and with a fixed composition so that it contained 10 MJ in total (see below). On the second day of study the subject came to the laboratory in the morning after having fasted for 12–14 h. They rested under standard conditions whereupon the resting oxygen consumption and carbon dioxide elimination were measured for 15 min. Immediately thereafter the subjects performed 36 W for 30 min on a bicycle ergometer and the respiratory gas exchange was measured during the last 15 min of the period. A test meal of 4 MJ (see below) was then given and the subjects rested horizontally for 30 min after which work and measurements were performed as before the test meal. The remaining part of the day was spent with ordinary activities but the food taken was restricted to that prepared in the laboratory. Additional energy intake with a fixed composition of 0–6 or 14 MJ was ensured. On the third day, the schedule of day 2 was repeated with the exception that the food intake after the test meal was free. Thus, measurements of oxygen consumption and carbon dioxide elimination were made on each subject at rest, during exercise before the test meal and during exercise after the test meal on different occasions, each consisting of consecutive days of which the first followed a day with an intake of 10 MJ and the second followed a day with an energy intake of 4, 10 or 18 MJ.

Work performance and respiratory gas exchange

The oxygen consumption and carbon dioxide elimination were measured using a low resistance Oth-McKerrow valve and the Douglas bag method. The bags were preinflated by the subject before the actual collection of expired air in order to ensure that the dead space in the bags was small and oxygen and carbon dioxide concentrations close to those resulting from the actual metabolism. Three bags were collected for each experimental period. The volume was measured in a spirometer and the oxygen and carbon dioxide concentrations were measured with a Servo oxygen analyser and a Beckman LBI carbon dioxide analyser respectively. Both instruments were calibrated using several gas mixtures with oxygen and carbon dioxide concentrations measured by the micro-Scholander technique (P. F. Scholander 1967). The expiratory air was dried immediately before being admitted to the analysers.

From every third Douglas bag, 5 ml samples were drawn and the concentrations of oxygen and carbon dioxide were also measured using the micro-Scholander technique. This procedure was used in order to check the stability of the instruments used for gas analysis. The mean value and standard deviation of the differences (instrument vs. micro-Scholander) for oxygen concentration were $\pm 0.05\%$ (absolute) and the corresponding values for carbon dioxide concentration were $\pm 0.05\%$ (absolute).

The Monark bicycle ergometer was calibrated before and several times during the experiment.

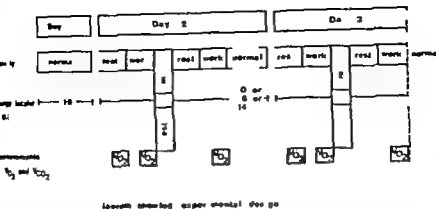


Fig. 1. The experimental design reported once a week over 3 weeks period. Day 1. The subject ate all the control meals at the laboratory (10 MJ), no measurements were performed. Day 2 and 3. V_O₂ and V_{CO}₂ were measured at the end of the rest period and work period before the test meal (4 MJ), and at the end of the work period after the meal. Between these two days measurements all meals are eaten at the laboratory. The energy contained in these meals are either 0-4 or 14 MJ.

according to the manufacturer's manual. The ordinary pendulum was replaced by a smaller pendulum 25 g in order to achieve greater sensitivity at the given work load. The subjects worked at 60 revolutions per minute as caused by a metronome and the work intensity was 36 W. Measurements of heart rate and respiratory frequency were performed both during rest and exercise. The heart rate was measured by ECG registrations with a digital display in combination with an ink-writer. The respiratory frequency was measured with a thermocouple transmitter placed in the valve close to the mouthpiece.

In 4 of the subjects, all experiments were performed with one week intervals on the same days in order to obtain daily physical activity. In one subject, all experiments were performed consecutively. The meals from this subject did not differ from those found in the other 4 subjects. All the subjects followed the same dietary schedule: first the 10-10 MJ experiment, then the 10-4 MJ and finally the 10-18 MJ experiment.

Composition and energy content of food

Every day food items commonly used in Denmark, a suitable number was selected which could easily be divided and weighed. 3 standard meals—breakfast, lunch and dinner—were prepared by commonly used cooking methods with the exception that no meat was fried with fat or oil. Each meal was weighed and its composition and energy content of each meal were calculated according to Heber (1973). The daily intake of 18 MJ was achieved by increasing the amount of each item in lunch and dinner by a factor of 1.2.

The energy content of one day's meals, from each of the 4, 10 and 18 MJ days, as determined by

Table 1. Composition and energy content of meals

	Composition, % of total energy as				Energy content, MJ	
	Carbohydrate	Fat	Protein	Alcohol	Calculated	Measured
Breakfast						
10 MJ	47.8	36.7	14.8	0	4.2	4.24
10 MJ	35.2	40.7	17.7	7.0	2.9	
10 MJ	42.6	37.3	14.1	6.0	3.4	
18 MJ	42.6	37.9	15.3	3.9	10.5	10.2
18 MJ	41.1	38.3	15.5	5.0	18.2	17.2
4 MJ	47.8	36.7	14.8	0	4.2	4.4

TABLE II Respiratory exchange ratio during exercise before and after the test meal on two consecutive days following an energy intake of 10 and 4 MJ and of 10 and 18 MJ

Subjects	Before the test meal						After the test meal					
	10	4	Δ	10	18	Δ	10	4	Δ	10	18	Δ
P J	0.746	0.711	0.035	0.762	0.720	-0.042	0.801	0.784	0.017	0.845	0.830	-0.015
S E D	0.796	0.777	0.019	0.849	0.858	+0.007	0.854	0.837	-0.017	0.863	0.881	+0.018
G H R	0.800	0.738	0.062	0.733	0.807	+0.064	0.848	0.804	-0.044	0.821	0.819	-0.002
A. B.	0.813	0.790	-0.023	0.842	0.846	+0.004	0.853	0.836	-0.017	0.876	0.890	+0.014
L. P.	0.827	0.796	0.031	0.783	0.810	+0.025	0.862	0.823	-0.039	0.835	0.859	-0.024
S. B.	0.798	0.746	0.052	0.811	0.814	+0.003	0.827	0.766	-0.061	0.843	0.832	-0.011
O. J.	0.810	0.741	-0.069	0.790	0.782	-0.008	0.848	0.829	-0.019	0.815	0.812	-0.003
Mean	0.799	0.754	0.044	0.796	0.805	+0.008	0.842	0.811	-0.031	0.842	0.846	+0.005
S.D.	0.026	0.028	0.019	0.042	0.045	0.032	0.021	0.027	0.018	0.022	0.031	0.017
S.E.	0.010	0.010	0.007	0.016	0.017	0.012	0.008	0.010	0.007	0.008	0.01	0.007

differences are also small, being 0 and $-18 \text{ ml O}_2 \text{ min}^{-1}$ for the effects before the test meal and 7 and $-6 \text{ ml O}_2 \text{ min}^{-1}$ after the test meal. None of the mean differences are statistically significant. Both before and after the test meal the oxygen consumption decreased on the day preceded by an intake of 18 MJ as compared with that on the day preceded by an intake of 4 MJ the values being -27 ± 20 and $-23 \pm 12 \text{ ml O}_2 \text{ min}^{-1}$ respectively.

The effect of the test meal on the oxygen consumption during exercise is also shown in Table II. The mean values before and after the test meal differ by about $50 \text{ ml O}_2 \text{ min}^{-1}$ in dependence on the previous day's intake.

The mean oxygen consumption during rest on two consecutive days following 10 and 4 MJ energy intake was $2.6 \text{ ml O}_2 \text{ min}^{-1}$ lower on the day following 4 MJ. On two consecutive days following 10 and 18 MJ it was $2.8 \text{ ml O}_2 \text{ min}^{-1}$ ($n = 7$) higher on the day following 18 MJ.

The respiratory exchange ratio observed during exercise are shown in Table III. The differences due to the test meal are about 0.05 and highly significant. The differences due to the previous day's intake, 10 MJ versus 4 MJ are highly significant, the latter being smaller by 0.04 and 0.03 before and after the test meal respectively. No significant differences were observed when comparing the values followed by a dietary intake of 10 and 18 MJ.

Discussion

Water and Wisc (1975) measured the cost of light arm exercise before and after a test meal containing 3.9 MJ at 3 levels of the preceding day's energy intake: 4, 10 and 17 MJ. In 4 subjects the cost before the test meal was found to be independent of the previous day's intake and was 2.8, 2.4 and 2.6 kJ min^{-1} (mean values), respectively. However, after the test meal the mean values were 1.8, 2.3 and 3.0 kJ min^{-1} respectively and this effect of the preceding day's intake was statistically significant. Thus, the cost of the postprandial exercise decreased by 1.0 kJ min^{-1} or 36%, when the previous day's intake was low and it increased by 0.4 kJ min^{-1} or 15%, when the previous day's intake was high.

TABLE II Oxygen consumption (ml O₂ STPD min⁻¹) during exercise before and after the test meal on two consecutive days following an energy intake of 10 and 4 MJ and of 10 and 18 MJ respectively

Subject	Before the test meal							After the test meal						
	10 MJ	4 MJ	Δ (4-10)	10 MJ	18 MJ	Δ (18-10)	Δ (18-4)	10 MJ	4 MJ	Δ (4-10)	10 MJ	18 MJ	Δ (18-10)	(18-4)
P. J.	852	830	-22	839	894	+55	+64	947	931	-16	950	947	-3	16
S. E. D.	747	774	+27	756	680	-76	-94	786	776	-10	767	806	+39	+38
G. H. R.	896	869	-27	907	879	-28	+10	994	864	-30	924	934	+10	30
A. R.	773	776	+3	730	739	+9	-37	783	853	+70	762	806	+44	47
L. P.	810	827	+17	780	772	-8	-55	853	852	-1	832	797	-35	55
S. B.	750	785	+35	749	723	-26	-62	796	827	+31	835	806	-29	25
O. J.	931	899	-32	933	882	-51	-17	945	948	+3	974	903	-71	83
Mean	823	823	0	813	796	-18	-27	877	878	+1	863	857	-6	22
S.D.	73	48	27	81	88	42	52	89	70	34	88	68	42	33
S.E.	27	18	10	31	33	16	20	34	26	13	33	26	16	11

bomb calorimetry (IKA C 400, Janke und Kunkel AG Staufen i. Br. Germany) after homogenisation in Waring blender. The heat released in the calorimeter was corrected by subtracting 5.04 kJ for each gram of protein in the mixture.

The composition and energy content of the meals are shown in Table I.

Results

The values for oxygen uptake and respiratory exchange ratio (R) measured during rest and during work at 36 W were found to be very similar to those usually found in young apparently healthy male subjects and the average net efficiency of the work was 17.9%. Considering the relatively low work intensity this is a normal value, cf. Gaesser and Brooks (1975). Also the heart rate and the ventilation/oxygen consumption ratio were normal to the conditions used. During exercise, 60 min after the test meal, the heart rate was, on average, 12 beats per minute higher than during work before the test meal. This is in agreement with what has been found elsewhere, cf. Dagenais, Oriol and McGregor (1966).

The results of measurements of oxygen consumption on 2 consecutive days were as follows: (1) at rest 249 ml min⁻¹ ± 29 ml min⁻¹ on day two and 243 ml min⁻¹ ± 28 ml min⁻¹ on day three, (2) during exercise before the test meal 822 ml min⁻¹ ± 75 ml min⁻¹ on day two and 870 ml min⁻¹ ± 110 ml min⁻¹ on day three, (3) during exercise after the test meal 890 ml min⁻¹ ± 103 ml min⁻¹ on day two and 912 ml min⁻¹ ± 118 ml min⁻¹ on day three. The sum of the variations due to methodological error and day-to-day variation was calculated for each of the conditions of rest, exercise before the test meal and exercise after the test meal and found to be 4.5, 6.1 and 6.9% (coefficient of variation) respectively.

The effect of the preceding day's dietary energy intake on the oxygen consumption during exercise is shown in Table II where the actual value on one day is compared to the corresponding value on the previous day, the latter preceded by a dietary intake of 10 MJ. The differences are rather small and range between -76 and +70 ml O₂ min⁻¹. The mean

have no explanation for this discrepancy. Only the type of work, arm exercise or leg exercise, was different in the two studies. A feedback mechanism of the general type suggested by several authors, Apfelbaum *et al* (1971) and Miller and Wise (1975), is not seen to be operative under the conditions studied by us, *i.e.* acute perturbations in order of 6–8 MJ of the input. Although such perturbations are large with respect to "normal" input, they are very small in relation to the body energy stores, *i.e.* about 100 MJ if the signals for control of energy expenditure are related to the latter: a difference of only 1% may be too small to activate the mechanism.

Authors are indebted to Mrs B. Troenker for help and suggestions concerning the composition of meals.

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The findings of Miller and Wise suggest the existence of an important feed-back loop in the regulation of the total energy balance of the body. Thus, out of a surplus of 7 MJ, added to the total body energy content during one day of overeating, not less than 0.4 MJ of the dietary energy of the next day's intake could be dissipated by chemical or mechanical "uncoupling" if one assumes that light exercise of the type studied by Miller and Wise takes up 16 h of the day. Furthermore, not less than 1 MJ of a 2 MJ deficit, incurred by one day's energy-deficient diet, could be recovered by an increased efficiency of utilization of the next day's food energy.

The results obtained in the present work can be discussed in terms of the mechanism suggested by Miller and Wise. It is, however, necessary first to consider how the measured values of oxygen consumption can be converted into values for the energy transformed during the different periods of the study. The facts that the workload was light and that \dot{V}_{O_2} and \dot{V}_{CO_2} calculations were made from analyses of gas collected during the last 15 minutes of 30 min working periods make it reasonable to believe that the ventilatory exchange quotient represents a true value for whole body RQ. Furthermore, the observed mean value of the pulmonary ventilation rate at work of about 22 l/min supports this belief. The energy transformed in association with the consumption of one liter of oxygen depends on the pathways involved in the oxidation and, since different steady state respiratory exchange ratios were observed in the various periods, it is necessary to use different factors for this conversion.

Benedict and Carpenter (1909-1910) measured the heat production and respiratory gas exchange in adult subjects performing little or no muscular activity (300-500 ml oxygen per minute) and on a normal diet. A plot of the heat produced per liter of oxygen consumed vs the respiratory quotient shows a rather considerable scatter but a mean increase of the former of about 1.4 kJ per liter, or about 6%, can be estimated for the RQ interval between 0.75 and 0.85. This value is considerably higher than that calculated on the assumption of complete stoichiometric combustion of normal food, i.e. about 0.6 kJ per liter. The estimate would not be expected to vary significantly with moderate variations in protein intake and breakdown, cf. Schulz (1975), and we assume that it is also relatively independent of moderate variations in physical activity.

The present work shows that a change in the energy content of the preceding day's meal (4 MJ vs 18 MJ) was associated with a mean increase of the respiratory exchange ratio of 0.051 and 0.035 (see Table III) before and after the test meal, respectively. The mean decrease in the oxygen consumption under these conditions was 3.3 (823-796/27 ml O₂/min) and 2.4% (878-857/21 ml O₂/min), respectively (Table II). When these values are converted to changes in energy transformation and corrected for the difference in RQ, they become 0.2 and 0.3% respectively. If, when correction for differences in RQ is made, a value of 0.6 kJ per liter is used instead of the value of 1.4 kJ per liter (see above), the energy transformation rate becomes slightly lower after 18 MJ than after 4 MJ. These numbers are associated with the same relative standard errors as that of the mean decrease in oxygen consumption, i.e. 2.4 and 1.3%, respectively. Thus, the energy transformation at the same work intensity is practically independent of the preceding day's energy intake.

The present results are thus at variance with those presented by Miller and Wise (1975).

We have no explanation for this discrepancy. Only the type of work, arm exercise *vs.* leg/leg exercise, was different in the two studies. A feedback mechanism of the general type suggested by several authors, Apfelbaum *et al.* (1971) and Miller and Wise (1975), does not seem to be operative under the conditions studied by us, i.e. acute perturbations of the order of 6–8 MJ of the input. Although such perturbations are large with respect to the "normal" input, they are very small in relation to the body energy stores, i.e. about 600 MJ. If the signals for control of energy expenditure are related to the latter, a difference of only 1% may be too small to activate the mechanism.

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The results obtained in the present work can be discussed in terms of the mechanism suggested by Miller and Wise. It is, however, necessary first to consider how the measured values of oxygen consumption can be converted into values for the energy transformed during the different periods of the study. The facts that the workload was light and that V_{O_2} and V_E calculations were made from analyses of gas collected during the last 15 minutes of 30 min working periods make it reasonable to believe that the ventilatory exchange quotient presents a true value for whole body RQ. Furthermore, the observed mean value of pulmonary ventilation rate at work of about 22 l/min supports this belief. The energy transformed in association with the consumption of one liter of oxygen depends on the pathways involved in the oxidation and, since different steady state respiratory exchange ratios were observed in the various periods, it is necessary to use different factors for conversion.

Benedict and Carpenter (1909-1910) measured the heat production and respiratory exchange in adult subjects performing little or no muscular activity (300-500 ml O_2 /min) and on a normal diet. A plot of the heat produced per liter of oxygen consumed vs. the respiratory quotient shows a rather considerable scatter, but a mean increase of the former of about 1.4 kJ per liter, or about 6%, can be estimated for the RQ interval between 0.75 and 0.85. This value is considerably higher than that calculated on the assumption of complete stoichiometric combustion of normal food, i.e. about 0.6 kJ per liter. The estimate would not be expected to vary significantly with moderate variations in protein intake (breakdown, cf. Schulz (1975)) and we assume that it is also relatively independent of moderate variations in physical activity.

The present work shows that a change in the energy content of the preceding day's meal (4 MJ vs. 18 MJ) was associated with a mean increase of the respiratory exchange ratio 0.051 and 0.035 (see Table III) before and after the test meal, respectively. The mean decrease in the oxygen consumption under these conditions was 3.3% (823-796-27 ml O_2 /min) and 2.4% (878-857-21 ml O_2 /min), respectively (Table II). When these values are converted to changes in energy transformation and corrected for the differences in RQ, they become 0.2 and 0.3% respectively. If, when correction for differences in RQ is made, a value of 0.6 kJ per liter is used instead of the value of 1.4 kJ per liter (see above), the energy transformation rate becomes slightly lower after 18 MJ than after 4 MJ. The numbers are associated with the same relative standard errors as that of the mean decrease in oxygen consumption, i.e. 2.4 and 1.3%, respectively. Thus, the energy transformation rate at the same work intensity is practically independent of the preceding day's energy intake. These results are thus at variance with those presented by Miller and Wise (1971).

glomerular population. Observations on glomeruli in deeper cortical layers are therefore highly desirable.

The aim of the present study was to investigate whether micropuncture and pressure measurements could be made on deep cortical structure after surgical exposure. Preliminary experiments with a craniotomy preparation were unsuccessful. A sagittal cut through the cortex could be kept open by a thin teflon sheet, but at re-investigation after 1 to 7 days the cut surface was too densely covered with adherent fibrin and/or granulation tissue to permit identification of any cortical structures. At the suggestion of H. R. Ulfendahl acute exposure was attempted, and turned out to be technically feasible.

A preliminary report of this study was presented at the II European Colloquium on Renal Physiology (Auckland, Tander and Nees, 1977).

Methods

Experiments were performed on Wistar and Sprague Dawley rats of either sex with body weight 200–300 g. Both strains are obtained from Møllegaards SPP A/S, Møllegaardsvej, Copenhagen. The animals were housed in pairs (Vedvandsvej Fælleskøkken, Bergen), given sodium intake of approximately 2 mmol/day. Usually food—but not water—was withdrawn in the afternoon the day before the experiments. The Sprague Dawley rats and one group of Wistar rats were studied in Lacton (Protosoft, Hamburg) anaesthesia, given as single intraperitoneal injection of 120 mg/kg BW. A second group of Wistar rats was anaesthetized with Metobarbital (pentobarbital), using an arterial p injection of 40 mg/kg, and additional doses of 3–5 mg/kg if needed. The rats were tracheotomized and placed on a heated operating table, the rectal temperature being controlled at 37.0°–37.5°C. For blood sampling and for recording of arterial blood pressure (P) a polyethylene tube (PE30) was placed in the descending aorta or the lower abdominal aorta. A femoral artery catheter was inserted for arterial injections. A femoral vein catheter was inserted for saline infusion. The rats were given during the experiments, except for occasional flushing of the catheters with isotonic saline, usually less than 2 ml in each experiment. Varying amounts of saline used for flushing the kidney may have been absorbed from the peritoneum. However, the urine appeared concentrated, and urine flow was about 0.5 μ l/min.

Preparation of the kidney

The left kidney was exposed through a subcostal incision and the urinary renal artery and vein were clamped. While some renal nerve strands were severed during dissection, no attempt was made at complete denervation. The kidney was placed in a Petri dish with its dorsal aspect facing upward, and surrounded by cotton moistened in saline. The ureter was cannulated in some experiments. When the ureter was cut, the maintenance of peristaltic waves was checked.

A sharp scalpel 1–2 mm thick, knife-shaped slice with a diameter of about 5 mm was cut off from the dorsal aspect of the kidney (Fig. 1 A). For shortness, this procedure and the cortical defect produced by it will hereafter be referred to as corticotomy. The immediate profuse bleeding stopped spontaneously in the course of 1–3 min. Blood was removed by repeated flushing with isotonic saline and suction by micropipette. The kidney was then covered with warm mineral oil. If not drained off, "tubular fluid" would accumulate at the corticotomy under the mineral oil. In order to establish the origin of this fluid, 0.5 M NaCl was infused at a constant rate of 15 μ l/min in 4 experiments. Arterial blood, urine and fluid from the corticotomy were collected simultaneously and sodium concentrations determined by liquid ion-selective counter. Possible reflex from the pelvis into the corticotomy was tested by puncturing the renal pelvis and injecting 0.1 ml Evans blue solution.

Maintenance of flow at the exposed glomeruli was tested by injecting Lissamine green and watching its appearance in the microscope.

Micropuncture was performed with glass pipettes with sharpened tip of 2 to 5 μ m diameter filled with 0.5 or 0.1 M NaCl colored with Evans blue. Neutralization of the solution with NaHCO_3 seemed to prevent precipitation of Evans blue. The micropipette was mounted on a Leitz-Wetzlar micromanipulator and the punctures were performed under Wild M5 stereomicroscope with magnification of 75.

Capillary Pressure in Deep and Superficial Glomeruli of the Rat Kidney

By

K. AUKLAND, K. HEYERAAS TONDER and G. NÆSS

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Abstract

AUKLAND K., K. HEYERAAS TONDER and G. NÆSS. *Capillary pressure in deep and superficial glomeruli of the rat kidney* Acta physiol. scand. 1977 101 418-427

A new technique has been developed to make deep renal cortical structures in rats accessible for micropuncture. The left kidney is dissected free and immobilized in a Lucite cup. A keratinized slice, 1 mm thick and about 5 × 5 mm wide, is cut off from the dorsal aspect of the kidney. Blood oozing from the cut surface is removed by flushing with saline and suction by microsponges. The bleeding stops in 1-3 min and causes none or only transient fall in arterial pressure (P_A). Up to 40 glomeruli become visible and remain circulated for several hours, as shown by injection of dye or silicone rubber. Glomerular capillary pressures (P_G), measured with servocontrolled counter pressure (Wiederhielm), showed no consistent change with time and no correlation to P_A . Average $P_G \pm S.E.$ in mmHg (number of glomeruli in parentheses) were: Wistar rats (WR), Inactin anesthesia, 57.8 ± 1.4 (41); Nembutal anesthesia, 58.1 ± 1.3 (13). Sprague Dawley rats, Inactin, 58.1 ± 1.7 (14). In WR, P_G was lower in deep than in midcortical glomeruli: <0.4 mm below kidney surface, 57.9 ± 1.8 (20); 0.5-0.9 mm, 60.5 ± 1.5 (20); and >1.0 mm, 53.8 ± 2.5 (13). Pressure in Bowman's capsule, 11.2 ± 0.6 (30). The observed P_G is higher than previously reported on the Munich mutant strain of WR, and suggests that glomerular filtration equilibrium is not reached.

Key words. Bowman's capsule, glomerular capillary pressure, glomerular filtration, kidney circulation, renal hemodynamics

While previous indirect estimates of glomerular capillary pressure (P_G) ranged from 60 to 90 mmHg, direct micropuncture of superficial glomeruli in a mutant Wistar rat (Munich strain) has given values of about 45 mmHg (Brenner, Troy and Daugharty 1971; Blantz *et al.* 1972). As a consequence of the latter estimate, the net filtration pressure is low: filtration equilibrium is reached before the end of the average glomerular capillary and the glomerular filtration rate will be strongly dependent on glomerular blood flow (Deen, Robertson and Brenner 1972, 1974). However, recent P_G measurements of 62.6 mmHg in superficial glomeruli which may be occasionally observed on the ventral aspect of the kidney in Sprague Dawley rats (Kjellskog *et al.* 1975 a) have seriously questioned the validity of the Munich Wistar strain as a representative model. In any case, it is not known whether accessible superficial glomeruli are representative for the whole or a great part of the

glomerular population. Observations on glomeruli in deeper cortical layers are therefore highly desirable.

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A preliminary report of this study was presented at the II European Colloquium on renal Physiology (Auckland, Tønder and Neam, 1977).

Methods

Experiments were performed on Wistar and Sprague Dawley rats of either sex with body weight 200–300 g. All animals were obtained from Møllegaards SPP Avlsboretorium, Copenhagen. The animals were housed on rat pellets (Vetenskaps Förlägg, Bergen), given sodium intake of approximately 2 mmol/day. Usual food—but not water—was withdrawn in the afternoon the day before the experiments. The Sprague Dawley rats and one group of Wistar rats were studied as intact (Proxonta, Hamburg) animals, given a single intraperitoneal injection of 120 mg/kg BW. A second group of Wistar rats was anesthetized with Mebrominal (pentobarbital), using an initial p injection of 40 mg/kg, and additional x doses of 3–5 mg/kg if needed. The rats were tracheostomized and placed on a heated operating table, the rectal temperature being controlled at 37.0°–37.5°C. For blood sampling and for recording of arterial blood pressure (P) a polyethylene tube (PE50) was placed in the aortic artery or the lower abdominal aorta via femoral artery. A femoral vein catheter was inserted for injections.

No fluid was given during the experiments except for occasional flushing of the catheters with isotonic saline, usually 0.5 ml below 2 ml in each experiment. Varying amounts of saline used for flushing the kidney may have been absorbed from the peritoneum. However, the urine appeared concentrated, and urine flow was low (5 μ l/min) before measurement.

Exposure of the kidney

The left kidney was exposed through a subcostal incision and the ureter, renal artery and vein were clamped. While some renal nerve strands were severed during dissection, no attempt was made at complete dissection. The kidney was placed in a Lucase cup with its dorsal aspect facing upward, and stabilized by cotton mounted on a suture. The ureter was cannulated in some experiments. When the ureter was in contact, the maintenance of peristaltic waves was checked.

A long sharp scalpel 1–2 mm thick, knife-shaped blade with a diameter of about 5 mm was cut off from the dorsal aspect of the kidney (Fig. 1A). For shortness, this procedure and the cortical defect produced by it will hereafter be referred to as corticotomy. The immediate profuse bleeding stopped spontaneously in the course of 1–3 min. Blood was removed by repeated flushing with isotonic saline and suction by micropipettes. The kidney was then covered with warm mineral oil. If not drained off, "tubular fluid" would accumulate in the corticotomy under the mineral oil in order to establish the origin of this fluid, 0.9% NaCl was infused at a constant rate of 15 μ l/min in 4 experiments. Arterial blood, urine and fluid from the corticotomy were collected simultaneously and osmotic concentrations determined in a vapor pressure osmometer. Possible reflex from the pelvic lobe to the corticotomy was tested by puncturing the renal pelvis and injecting 0.2 ml Evans blue solution.

Maintenance of flow in the exposed glomeruli was tested by injecting Evan's blue and watching its appearance in the micropipette.

Micropuncture was performed with glass pipettes with sharpened tip of 2 to 3 μ m diameter filled with 0.1 M NaCl colored with Evans blue. Neutralization of the solution with NaHCO₃ seemed to prevent precipitation of Evans blue. The micropipette was mounted in a Leitz Wetzlar micromanipulator and the punctures were performed under Wild M5 stereomicroscope with magnification of 75.

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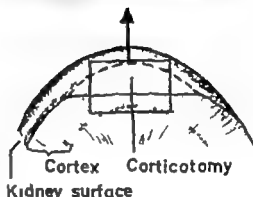
Abstract

AUKLAND K., HEYERAAS TØNDER K. and NÆSS G. *Capillary pressure in deep and superficial glomeruli of the rat kidney*. Acta physiol. scand. 1977 101 418-427

A new technique has been developed to make deep renal cortical structures in rats accessible for micropuncture. The left kidney is dissected free and immobilized in a lucite cup. A lens-shaped slice, 1.2 mm thick and about 5-5 mm wide, is cut off from the dorsal aspect of the kidney. Blood oozing from the cut surface is removed by flushing with saline and suction by microsponges. The bleeding stops in 1-3 min and causes none or only transient fall in arterial pressure (P_A). Up to 40 glomeruli become visible and remain circulated for several hours, as shown by injection of dye or silicone rubber. Glomerular capillary pressures (P_G), measured with servocontrolled counter pressure (Wiederhielm), showed no consistent change with time and no correlation to P_A . Average $P_G \pm S.E.$ in mmHg (number of glomeruli per parentheses) were: Wistar rats (WR), Isoactin anesthesia, 57.8 ± 1.4 (41), Membromal anesthesia, 58.1 ± 1.1 (13), Sprague Dawley rats, Isoactin, 58.1 ± 1.7 (14). In WR, P_G was lower in deep than in midcortical glomeruli: <0.4 mm below kidney surface, 57.9 ± 1.8 (20), 0.5-0.9 mm, 60.5 ± 1.5 (20) and >1.0 mm, 53.8 ± 2.5 (13). Pressure in Bowman's capsule, 11.2 ± 0.6 (30). The observed P_G is higher than previously reported on the Munich mutant strain of WR, and suggests that glomerular filtration equilibrium is not reached.

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2. Kidney exposed with silicone rubber or other corticotomy. Transverse section with corticotomy made after dehydration during with methyl benzoate. Ventralside 1.38" above the long axis of the kidney with under corticotomy layer because section.

Results

Verification of the preparation

described above the corticotomy caused only shortlasting bleeding, and the total blood loss was estimated at 0.5 ml or less. Uncontrolled bleeding did not occur in any of 40 expts. arterial pressure (P_A) remained unchanged or fell transiently by at most 15 mmHg for a total of 1 to 5 min.

The corticotomy might expose as many as 40 glomeruli, and injection of dye and silicone rubber indicated that blood flow was maintained in practically all visible glomeruli (Fig. 2). It was also the case with glomeruli located so close to the incision that they could be seen to protrude beyond the cut surface. Since these glomeruli were loose and therefore liable to puncture, most measurements were made on glomeruli at an approximate depth of 50 to 100 μ m under the corticotomy. The depth of the punctured glomeruli relative to the kidney surface was approximated by placing a fine forceps with 0.3 mm broad teeth exactly in the corticotomy and estimating the position of the ablated cortical surface under low magnification. The most superficial glomeruli were located 0.15 to 0.20 mm under the capsule, while the deepest punctured glomeruli had a depth of 1.3-1.5 mm. The silicone rubber preparations indicated that some juxtamedullary glomeruli—giving rise to arterioles recta—were exposed and might have been punctured. However since the morphology of the punctured glomeruli was not studied, the present measurements do not warrant any conclusion concerning pressure in juxtamedullary glomeruli versus deep glomeruli of the cortical type.

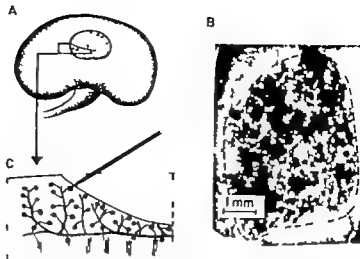


Fig 1 Corticotomy as viewed from above, A Schematically and B Photographed in vivo. Glomeruli more prominent than in hydroponic kidneys after intravenous infusion of 7 ml isotonic saline. C. Sagittal section through corticotomy as indicated in A. Schematic picture of arcuate and interlobular arteries, afferent arterioles and glomeruli.

Pressures were recorded continuously with Hewlett Packard pressure transducers (Model 1280 C) connected to a Hewlett Packard recorder (Model 8811 A). The micropipette measurements were obtained by means of a servo-controlled counterpressure ad modum Wiederholm *et al* (1964), with a setup slightly modified from that described by Iagiletti, Pawula and Tompkins (1970). (See Acknowledgement at the end of the paper). The transducers were calibrated before each expt., and zero pressure was checked repeatedly by placing the micropipette in a refilled cup at kidney level, or in the "tubular fluid" accumulated in the corticotomy. Since the location of the micropipette with a glomerular capillary cannot be ascertained visually, one has to rely on indirect criteria for acceptance of pressure measurements.

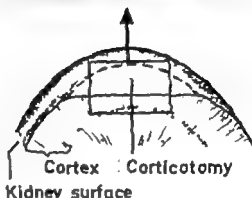
1. The recorded pressure should not change when the feedback gain of the servocontrolled counterpressure system is varied. Because the feedback signal consists of the electrical resistance within and at the opening of the pipette tip, artificially high pressures are obtained if the pipette tip hits against the vessel wall, and the vessel mate will increase with increasing feedback gain.
2. The systolic pressure peak should coincide with or follow within a few milliseconds that of the arterial pressure curve.
3. Pulse-to-pulse and second-to-second variations of arterial pressure should be paralleled in the glomerular capillary pressure.
4. The glomerular pulse pressure curve should reflect characteristics of the arterial pressure, e.g. the diastolic notch.
5. Injection of dye should not appear in Bowman's space, tubules or interstitium.

While the first two criteria and in general also 3 were satisfied in the reported recordings, criterion no. 4 was not always present and no 5 was not tested systematically. However, pressure recordings deemed absolutely ideal according to the mentioned criteria, did not differ from the rest of the accepted measurements.

The position of the pipette in Bowman's capsule was verified by the appearance of injected dye in the proximal tubuli.

At the end of 3 expts. silicone rubber (Microfil MV 112, Cation Bio-Medical Products, Boulder Colorado) was injected into the abdominal aorta. When the superficial renal vessels seemed filled, the kidney was clamped and excised. After dehydration in ethanol of increasing concentrations the organ was cleared in methyl benzoate.

Plasma colloid osmotic pressure was measured directly in arterial blood samples taken at the time of micropuncture by means of the membrane osmometer described by Åstrand and Johnsen (1974). Unfortunately many samples were lost during storage and reliable measurements were obtained in only 10 animals.



Kidney exposed with silicone rubber after corticotomy. Transverse section of corticotomy made after dehydration using 10% methyl benzoate. Viewpoint 1.30 above the long axis of the kidney took under corticotomy body because silicon.

Results

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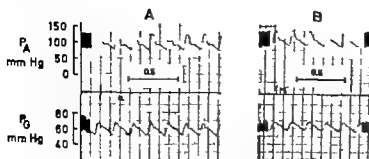


Fig. 3 Original recording of arterial (P_A) and glomerular capillary (P_G) pressures. Glomerulus located 0.6 mm (A) and 0.1 mm (B) under kidney surface.

No measurements were made until at least 15 min after the corticotomy. While the surface often appeared increasingly swollen throughout the expts., glomeruli remain clearly visible and might be punctured for at least 3 h after corticotomy.

Approximate estimates of the rate of fluid accumulation in the corticotomy in 4 rats gave values of 1–5 μ l/min. Apparent variations in flow rate, and the finding in 1 rat that dye injected into the renal pelvis appeared as a discrete spot deep in the corticotomy suggested that reflux of pelvic urine might occur at slight elevations of pelvic pressure. However, the suspicion was not confirmed by measurements of inulin concentration. Corticotomy fluid/plasma (CF/P) inulin ratios determined in 4 rats varied from 1.4 to 9 with corresponding urine/plasma (U/P) ratios of 188 to 800. Elevation of pelvic pressure 20 mmHg in one experiment increased CF/P from 1.4 to 5.8, while U/P increased from 2 to 450. In a second rat (saline diuretic), pressure elevation increased CF/P from 3.3 to 3 and U/P from 60 to 82. These observations would seem to exclude appreciable reflux of pelvic urine, and rather indicate that the corticotomy fluid is mainly derived from severe proximal tubules, with some slight admixture from more distal nephron segments. This conclusion also agrees with the finding that dye injected into Bowman's space usually emerged in the corticotomy after traversing one or several proximal tubular loops.

Pressure measurements

Satisfactory measurements were obtained in 1 to 7 glomeruli in each rat. Examples of original P_G recordings on subcortical glomeruli are shown in Fig. 3 and 4. As evident from Fig. 4, short-lasting spontaneous variations in P_A were faithfully reflected in P_G , but with a tendency to relatively greater excursions in P_G . A more direct evidence of rapid changes in renal hemodynamics was observed after bolus injection of saline (Fig. 4). The

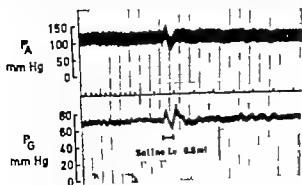


Fig. 4 Original recording of arterial (P_A) and glomerular capillary (P_G) pressures. Glomerulus located 0.4 mm under kidney surface. Time mark: g 10.

Table 1. Pressures in aorta (P_A), glomerular capillaries (P_G) and Bowman's capsule (P_B). Mean \pm S.E.

	N ^a	P_A	P_G	P_B
Water, Inactin	18	104.2 \pm 2.3 (41)	57.8 \pm 1.4 (41)	12.6 \pm 1.4 (9)
Water, Metbionin	4	108.9 \pm 2.7 (13)	58.1 \pm 1.3 (13)	10.5 \pm 2.0 (10)
Sprague Dawley Inactin	8	109.9 \pm 3.0 (14)	58.1 \pm 1.7 (14)	10.7 \pm 1.1 (11)

^a Number of rats. Number of measurements in parentheses. Pressures in mm Hg.

secondary fall in P and P_G was followed by an inproportionate rise in P_G , suggesting dilation of the afferent arteriole as response to the transient pressure reduction.

Provided satisfactory pipette position was maintained, the recorded P_G would usually remain unchanged from the moment of puncture and for a period of up to 30 min. However in some cases the pressure recorded immediately after puncture might be low e.g. 40-45 mmHg, and would then rise to a steady level of 55 to 65 mmHg in the course of 1 to 1.5 sec. In such cases the steady pressure has been presented in figures and tables.

As shown in Table 1, average P was practically identical in Wistar rats anesthetized with Inactin or with Metbionin, 57.8 and 58.1 mmHg respectively and a similar average (58.1 mmHg) was observed in Sprague Dawley rats anesthetized with Inactin. No difference was observed between punctures made early (15-60 min) or 1-3 h after corticotomy.

As evident from Fig. 5 P showed no correlation to spontaneous intra- and inter-individual variations in the arterial pressure level. Furthermore, as shown in Fig. 6, there was no marked difference in P between deep and superficial glomeruli. An arbitrary division of glomeruli in three groups according to depth under the kidney surface confirmed this general impression (Table II). However in the Wistar rats the average P_G in the deepest glomeruli (53.8 mmHg) was significantly lower ($p < 0.02$) than that of mid-cortical glomeruli (60.5 mmHg). The pressures in Bowman's capsule ranged from 6 to 20 mmHg, with an average for all groups of rats of 11.2 ± 0.6 (S.E.) mmHg (Table I).

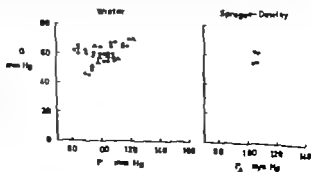


Fig. 5. Glomerular capillary pressure (P_G) in 22 Wistar and 8 Sprague Dawley rats related to corresponding mean arterial pressure (P_A). Open and filled circles: Inactin anesthesia. Triangles: Metbionin anesthesia.

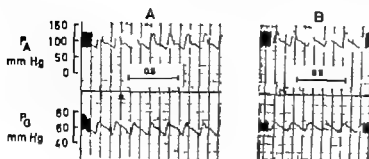


Fig. 3. Original recording of arterial (P_A) and glomerular capillary (P_G) pressures. Glomeruli located 0.6 mm (A) and 0.8 mm (B) under kidney surface.

No measurements were made until at least 15 min after the corticotomy. While the cortex surface often appeared increasingly swollen throughout the experiments, glomeruli remained clearly visible and might be punctured for at least 3 h after corticotomy.

Approximate estimates of the rate of fluid accumulation in the corticotomy in 6 rats gave values of 1–5 μ l/min. Apparent variations in flow rate, and the finding in one rat that dye injected into the renal pelvis appeared at a discrete spot deep in the corticotomy suggested that reflux of pelvic urine might occur at slight elevations of pelvic pressure. However, the suspicion was not confirmed by measurements of inulin concentration. Corticotomy fluid/plasma (CF/P) inulin ratios determined in 4 rats varied from 1.4 to 9 with corresponding urine/plasma (U/P) ratios of 188 to 800. Elevation of pelvic pressure 20 mmHg in one experiment increased CF/P from 1.4 to 5.8 while U/P increased from 21 to 450. In a second rat (saline diuresis), pressure elevation increased CF/P from 3.3 to 3.8 and U/P from 60 to 82. These observations would seem to exclude appreciable reflux of pelvic urine, and rather indicate that the corticotomy fluid is mainly derived from several proximal tubules, with some slight admixture from more distal nephron segments. This conclusion also agrees with the finding that dye injected into Bowman's space usually emerged in the corticotomy after traversing one or several proximal tubular loops.

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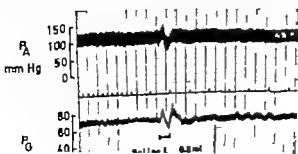


Fig. 4. Original recording of arterial (P_A) and glomerular capillary (P_G) pressures. Glomerulus located 0.4 mm under kidney surface.

and we therefore tend to reject technical-methodological bias as explanation for the high pressures observed in the present rats compared to those reported in the "Munich strain".

In discussing the physiological meaning of the observed glomerular pressures one should keep in mind that all measurements were made 15 to 180 min after surgical intervention on the kidney which might interfere with local hemodynamics in various ways:

The apparent swelling of the cut surface might reflect an increased local interstitial pressure which might dilate the afferent arterioles through a myogenic response to reduced transmural pressure (for references see Aukland 1976). The finding of "normal" pressures in Bowman's space speaks against a large increase in tissue pressure, but obviously a more complete characterization of the preparation demands studies on autoregulation of glomerular capillary pressure, blood flow and filtration rate.

The corticotomy might well lead to local release of vasoactive substances such as histamine, serotonin, plasmakinins, prostaglandins or renin/angiotensin, but we have no evidence to decide to what extent and in what direction such factors may have influenced the measured P_G . Since inflammatory processes usually have a marked phasic pattern, the observation that P did not change systematically with time after corticotomy would to some extent speak against strong influence of local vasoactive substances.

Possible reflux of urine from the renal pelvis into the corticotomy might flood the cortical incision with a hyperosmolar solution and give asodilation. While such reflux was not completely excluded, the finding of corticotomy fluid/plasma osmotic ratios of less than 10 and an increased ureteral pressure seemed to exclude appreciable hyperosmolarity.

The observation that most proximal tubules corresponding to the exposed glomeruli were severed by the corticotomy implies that a possible tubuloglomerular feedback mechanism ("macula densa feedback") is disrupted. However, judged from studies on peripheral nephroses little change in P should be expected. The reflex is mainly activated by decreasing distal tubular flow rate,—leading to a fall in P_G —whereas reduction of flow rate to normal level has little or no effect (Schniermann, Persson and Ågerup 1973; Hierholzer *et al.* 1974).

Probably of greater hemodynamic significance, many interlobular arteries have been cut and sealed by thrombi. Since in most experiments a lens-shaped corticotomy was made, many of the punctured superficial glomeruli might derive their afferent arteriole from cut interlobular arteries, whereas deeper glomeruli most likely would be fed from totally amputated interlobular arteries (Fig. 1 and 2). To the extent that there normally is appreciable resistance, and pressure fall, from the arcuate arteries to the origin of deep and subcortical afferent arterioles, the amputation and resulting flow reduction in the interlobular arteries should lead to an increased pressure in the remaining portion of the vessels. Such "reversed steal effect" of amputation might well be compensated by autoregulatory increased resistance in the afferent arterioles, but if that is not the case, the tendency to lower pressure in deep than in superficial glomeruli (Fig. 6, Table II) might be more pronounced in the intact kidney.

The strongest evidence that "normal" glomerular pressures are obtained with the present technique is the good agreement with the pressures obtained in Sprague-Dawley rats in functional superficial glomeruli punctured through the intact kidney capsule, averaging

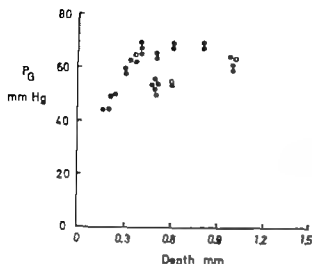


Fig. 6. Capillary pressures (P_G) in glomeruli at various depths under kidney surface in Wistar (closed circles) and 8 Sprague Dawley rats (open circles).

Plasma colloid osmotic pressure at the time of micropuncture averaged 14.3 mm (n=10). This is considerably lower than the 16–20 mmHg previously observed in Wistar rats shortly after Mebumal anesthesia (Johnsen 1974 and unpublished results).

Discussion

The observed glomerular capillary pressures agree well with those recorded by Kallik *et al* (1975a) on occasionally occurring superficial glomeruli in Sprague Dawley rats, but are 10–20 mmHg higher than those observed in several studies on the mutant Muni strain of Wistar rats (Brenner *et al* 1971; Blantz *et al* 1972). While it is tempting to ascribe the discrepancy solely to differences between rat strains, possible methodological differences should be considered. We believe that the criteria for acceptable measurements set out above are sound, and technically our recordings would seem to be well comparable to the few previously published records of glomerular pressures. We have been aware that inadequate positioning of the micropipette within the capillary lumen may result in too high values, and high pressure records have therefore been considered with special suspicion. However, many pressures of 60–70 mmHg were obtained in technically "ideal" recordings.

TABLE II. Glomerular capillary pressure (mmHg) at varying cortical depths. Number of glomeruli in parentheses.

	Depth under cortical surface		
	<0.4 mm	0.5 to 0.9 mm	1.0 mm
Wistar	57.9 ± 1.8 (70)	60.5 ± 1.5 (20)	53.8 ± 2.5 ^a (13)
Sprague Dawley	62.8 ± 2.4 (5)	54.9 ± 2.1 (7)	58.0 (2)

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62.6 mmHg (Källskog *et al* 1975 a). Indirect estimates from proximal tubular stop flow pressure have given varying results, but several investigators have arrived at even high P_O than that observed here (Källskog *et al* 1975 a, Brenner *et al* 1971 and references in the articles).

A P_O of about 45 mmHg, as observed in Munich Wistar rats, indicates that filtrate equilibrium is reached at or just before the end of the glomerular capillaries, i.e. the plasma oncotic pressure, increased by ultrafiltration balances the transcapillary hydrostatic pressure difference (Brenner *et al* 1971, Blantz *et al* 1972). As a consequence, glomerular filtration rate will be strongly influenced by glomerular blood flow (Deen *et al* 1972, 1974). In the present study pressures of 45 mmHg or lower were observed in only 5 out of 10 glomeruli. While the filtration fraction was not measured, it seems likely that a net filtration pressure of 10 to 15 mmHg would remain at the efferent end of the capillaries in most glomeruli, and blood flow variations should only modestly influence GFR (Källskog *et al* 1975 b).

The main new information obtained in the present study would seem to be the similarity of P_O in deep and superficial glomeruli. While in Wistar rats the deepest glomeruli had lower pressure than midcortical glomeruli (Table II, Fig. 6), the difference was small, and we would hesitate to attach much significance to these numbers. Obviously it would be of interest to record P_O in deep glomeruli identified as being of the juxtamedullary type.

The apparently well preserved blood flow and pressure in the exposed glomeruli suggest that the corticotomy technique could be used for micropuncture studies on other deep cortical structures. While not explored so far, interlobular vessels might be identified for pressure measurements, and studies on tubular functions might well be feasible. The technique is obviously traumatic, but is probably closer to physiological conditions than *in vitro* studies on homogenates, slices, isolated cells or tubules.

We are greatly indebted to Hans R. Ulfendahl and Mats Wolgast, Biomedicum, Uppsala, 5 edn, for patient instruction in micropuncture technique and generous lending of equipment.

We also wish to thank laboratory engineer Erik O. Halleland, Institute of Physiology, Bergen, Norway for constructing the mechanical parts of the servo pressure apparatus, and electroengineer Bjørn Hovden, Laboratory for Electronics, Preclinical Institutes, Bergen who modified and constructed the electronic feedback system. The modification of the Intaglietta model mainly included more extensive use of integrated components. Detailed description may be provided on request to the authors.

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Catecholamines and Pancreatic Hormones during Autonomic Blockade in Exercising Man

By

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Abstract

GALDO H., N. J. CHRISTENSEN and J. J. HOLST: *Catecholamines and pancreatic hormones during autonomic blockade in exercising man*. Acta physiol. scand 1977 101 428-4.

The importance of autonomic nervous activity for the pancreatic hormonal response to exercise in man studied 7 men ran at 58% of \dot{V}_{O_2} max (determined without administration of drugs): exhaustion during α -adrenergic blockade with phentolamine (P), during parasympathetic blockade with atropine (A), or without drugs (C). At rest phentolamine increased the plasma concentrations of both insulin and norepinephrine. During exercise norepinephrine concentrations increased and were in P experiments 3 times the concentrations in C experiments. Insulin always declined during exercise but in P experiments never decreased below basal levels. At identical times neither glucagon nor glucose differed significantly in the different experiments. Thus during exercise α -adrenergic blockade increased insulin concentrations but did not diminish glucagon response. Nor was this response increased when β -receptor stimulation in P experiments intensified by the particularly high catecholamine concentrations. The concentrations of FFA, glycerol and lactate were highest in P experiments and identical in A and C experiments. These findings indicate that during prolonged moderate exercise in man insulin secretion is depressed by stimulation of α -adrenoreceptors whereas glucagon secretion is not influenced by adrenergic receptors. Stimulation of β -adrenoreceptors enhances lipolysis but neither lipolysis nor pancreatic hormonal secretion is influenced by cholinergic activity during exercise.

Key words. Exercise, epinephrine, norepinephrine, glucagon, insulin, sympatholytics, atropine, phentolamine, autonomic nervous system, fatty acids.

Current evidence suggests that in several species stimulation of the vagus nerve increases both insulin and glucagon secretion whereas stimulation of the sympathetic pancreatic nerve diminishes insulin release and enhances glucagon release. A basal tonic discharge from the sympathetic as well as the parasympathetic nervous system has been proposed to influence the pancreatic A and B cells in the postabsorptive state, and an increased discharge possibly modulates the function of these islet cells during hypoglycemia. However, the role of neural mechanisms in the physiologic regulation of insulin and glucagon secretion is unsolved (for recent reviews see Day 1975, Gerich, Charles and Grodsky 1976, Unger and Orci 1977, Woods and Porte 1974).

During exercise the activity of the autonomic nervous system changes. Vagal tone, at least at the heart, is reduced (Ekblom *et al.* 1972), and the net activity in the sympathetic nervous system as reflected in plasma catecholamine levels is increased (Galbo, Holst and Christensen 1975). The decrease in plasma glucose concentrations which often occurs during prolonged submaximal exercise (Galbo *et al.* 1976, Galbo, Christensen and Holst 1977, Harvey, Faloon and Unger 1974) may contribute to an activation of autonomic centers in the brain. In the α -adrenergic blocking agents were able to influence the hormonal response to exercise (Harvey, Faloon and Unger 1974, Luyckx and Lefebvre 1974). While α -adrenergic blocking drugs abolished the exercise-induced decrease in plasma insulin concentrations (Harvey, Faloon and Unger 1974, Luyckx and Lefebvre 1974), the exercise induced increase in glucagon concentrations has been abolished by α - (Harvey *et al.* 1974, Luyckx and Lefebvre 1974) as well as by β - (Luyckx and Lefebvre 1974) adrenergic blocking drugs. Thus the pancreatic hormonal response to prolonged exercise, i.e. a decrease in plasma insulin concentrations and an initial decrease followed by an increase in glucagon concentrations, was modified by β -adrenergic blockade (Galbo *et al.* 1976, 1977). To further elucidate the importance of autonomic nervous activity for the pancreatic hormonal response to exercise *in man*, we have now measured plasma concentrations of glucagon, insulin, catecholamines and substrates during prolonged exercise which was performed after administration of either α -adrenergic blocking drug phentolamine, or the anticholinergic drug atropine, or no drugs. The stimulation of cholinergic receptors may inhibit (Colville *et al.* 1964, Weiss and Lusk 1965) and the stimulation of adrenergic receptors promote (Day 1975) lipolysis in tissue. The administration in the present study of autonomic blocking agents allowed us to examine also the influence of the autonomic nervous system on lipolysis during exercise.

Methods

Forty healthy male students 24 (20–29) yr (mean and range) volunteered in the study. The same subjects had also the previous 5 months participated in similar studies in our laboratory (Galbo *et al.* 1976) and are accordingly familiar with the applied procedures. Their mean maximal \dot{V}_{O_2} uptake ($\dot{V}_{O_{2max}}$) determined using breathal analysis (without preceding administration of drugs) was 4.27 (3.39 – 5.17) l min^{-1} and 5.14 – 6.0 $\text{ml kg}^{-1} \text{ min}^{-1}$. Mean weight was 77 (63 – 90) kg and mean height 184 (167 – 194) cm. Each subject participated in three exercise programs in random order and at intervals of 3 (2–5) weeks. The subjects ate and slept throughout the study and avoided physical training for three days before each exercise program. The subjects arrived in the laboratory at 8 o'clock in the morning after good night sleep and at least 10 h fast and tobacco abstinence, in addition to at least 37 h alcohol abstinence. They were weighed and had an indwelling catheter inserted in the forearm, through which they fasted as clear as possible. The heart rate was registered with surface electrodes, both as an electrocardiogram and as a cardiotelemetry. The cardiogram and the ECG were continuously displayed on an oscilloscope and periodically recorded throughout the exercise. While the subjects were standing, their expired air was collected in Douglas bags and blood samples were drawn without stress. Both gas and blood samples were designated as (rest). The expired air was analysed with an infrared CO_2 -analyser (Beckman LB-1, QA 184) and paramagnetic O_2 analyser (Servomox). The accuracy of the analysis as verified with the Scholander microtechnique. On morning the subjects then had 0.035 mg kg^{-1} b wt of atropine (A) injected in 5 min 10 min after the start of the exercise expired air was collected and blood samples were drawn (rest, atropine). During the subsequent exercise additional 0.035 mg kg^{-1} b wt of atropine were injected every 70 min. During exercise the subjects rinsed out their mouth repeatedly with water, while overheating was avoided by aid of fan and wet towels. 36 min after exercise the persistence of cholinergic blockade was tested by clinical examination and further verified by the lack of effect of 15 mg of metacholine administered i.v.

Another morning the subjects received the dose of phentolamine usually applied in order to establish an α -adrenergic blockade (Reinheimer, Davidson and Albrink 1968). Immediately after rest-samples had been drawn, 5 mg of phentolamine (P) was administered i.v. followed by an i.v. infusion of 0.5 mg/min of phentolamine dissolved in isotonic sodium chloride (0.67 ml/min). After 40 min of infusion expired air was collected, and blood samples were drawn (rest₂-samples). The subjects began to run, and the infusion was maintained, until the last blood samples had been drawn.

In a control-expt. (C) the subjects began to run immediately after the first blood samples had been drawn.

Exercise was performed on a motor-driven treadmill, the speed and inclination of which for each subject were identical in the different experiments. The work load was designed to approximate 60% of individual maximal oxygen uptake determined in separate expts. without administration of drugs. Immediately after the last blood samples had been drawn at rest (rest₂) the subjects began to run during repeated 30 min bouts separated by 10 min rest intervals. This sequence was continued until exhaustion. Expired air was collected through a mask after 20 min of each exercise period. In each exercise period, venous blood samples were drawn after 15 min for analysis of glucose, insulin and glucagon, and during the last 2 min for analysis of glucagon, insulin, catecholamines, glucose, glycerol, FFA (free fatty acids), lactate, and hematocrit. Finally blood samples and expired air were collected 30 min after exhaustion. Minors water was offered when desired.

The methods of sampling and analysis of blood have been reported elsewhere (Galbo *et al.* 1970). In respect to glucose concentrations arterialized capillary blood obtained from finger tips—as found not to differ significantly from blood in the cannulated forearm vein. Furthermore, the oxygen saturation of the sampled venous blood was higher than 90% throughout the experiments. These findings indicate that the use of arterial instead of venous samples would not have changed the results. Every subject had all analyses of a hormone carried out in a single assay run and the radioimmunoassays were carried out with antibodies from the same batch as in our preceding exercise studies. Statistical evaluation of the data was made by means of correlation analysis and by means of Wilcoxon's nonparametric ranking test and the *t*-test for paired comparisons (Snedecor and Cochran 1965). Differences were considered to be significant, if *p* value of less than 0.05 were obtained with both these tests. When nothing else is stated, concentrations obtained at exhaustion (*n* = 21) have been used for correlation analysis. The cited correlation coefficients are significant on the five per cent confidence level.

Results

None of the basal values (rest) differed significantly in the three experiments. At rest atropine increased heart rate and serum alanine concentrations and decreased plasma glucose concentrations whereas phentolamine increased heart rate as well as the concentrations of norepinephrine, insulin, FFA, glycerol and lactate (Fig. 1–5). The increments at rest in norepinephrine and insulin concentrations correlated significantly (*r* = 0.95).

During exercise oxygen uptake (57.1 ± 1.5 (A), 58.1 ± 1.3 (P), 57.5 ± 1.3 (C)) of individuals ($V_{O_2 \max}$, mean and S.E.) as well as metabolic rate (kcal/min) (as calculated from oxygen uptake and R value (respiratory exchange ratio)) were identical during all exercise bouts. The total work time, however, was significantly longer in control expts. (175 ± 7 min, mean and S.E.) than in atropine expts. (111 ± 12 min) and phentolamine expts. (108 ± 12 min). Apart from a marked dryness of the mouth in all atropine expts. the subjective experience of exercise varied from individual to individual. In atropine expts. palpitations, irritability, nausea and dizziness were reported. In phentolamine expts. palpitations, oppression, dyspnoea, increased tendency to sweating, and pain in the calves were occasionally reported, whereas dizziness was not experienced during exercise. At exhaustion in nearly all experiments the subjects reported a feeling of generalized fatigue.

At identical times during exercise the glucagon concentrations in plasma did not differ significantly in the three experiments (Fig. 1). After an initial decrease glucagon concentrations subsequently increased progressively in each expt. At exhaustion glucagon concentra-

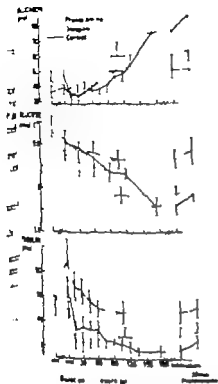


Fig. 1

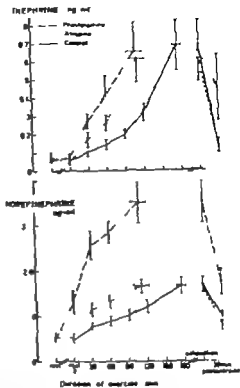


Fig. 2

Mean concentrations (\pm S.E.) of glucagon, glucose and insulin in plasma obtained during rest and prolonged exercise are plotted at the work time. When rest, samples had been drawn, drugs were administered. 7 μ g/kg of insulin equals one μ U/ml².

Mean concentrations (\pm S.E.) of plasma catecholamines obtained during rest and prolonged exercise and at the work time. When rest, samples had been drawn, drugs are administered.

were higher in control (than in phentolamine expts. (Fig. 1). 30 min postexercise glucagon concentrations did not differ significantly from concentrations at exhaustion and were still at preexercise levels. Glucagon correlated significantly with glucose concentrations at rest ($r = 0.6$) and with decreases in glucose concentrations during exercise ($r = 0.6$). Increments in glucagon concentrations during exercise correlated with glucose concentrations at exhaustion ($r = 0.65$) and with decreases in glucose concentrations ($r = 0.6$). Glucagon and increments in glucagon concentrations during exercise correlated with epinephrine ($r = 0.5$ and 0.6 , respectively) but neither with norepinephrine nor with alanine, lactate and FFA concentrations. Insulin concentrations in plasma always declined during exercise (Fig. 1). During α -adrenergic blockade, however, insulin concentrations were significantly higher than in control expts. during as well as after exercise, and never decreased significantly below basal (rest) levels (Fig. 1).

Concentrations of catecholamines in plasma always rose throughout exercise and were at least 10 times lower in control expts. (Fig. 2). During as well as after exercise the con-

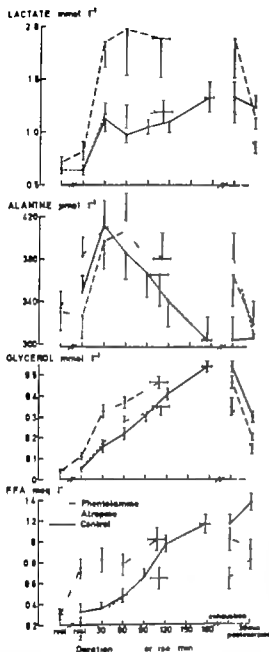


Fig. 3. Mean concentrations (\pm S.E.) of lactate, blood and of lactate, glycerol and free fatty acids in serum obtained during rest and prolonged exercise plotted at the work time. When rest samples been drawn, drugs were administered.

concentrations of norepinephrine were highest in phentolamine expts., while epinephrine concentrations at exhaustion were identical in the different expts. (Fig. 2). 30 min postexercise the catecholamine levels had decreased but were still higher than preexercise levels highest in phentolamine expts. (Fig. 2). In atropine and control expts. epinephrine correlated significantly with the decrease in glucose concentrations during exercise ($r = 0.65$).

The carbohydrate combustion rate (calculated from O_2 uptake and R value measurements) was during exercise significantly higher in atropine expts. than in control expts. (represented 55 and 46%, respectively of total caloric output per min (Fig. 4). Plasma glucose concentrations declined gradually during exercise and were during the first 60 min of run identical in the different expts. (Fig. 1). At exhaustion, however, the decrease in glucose c

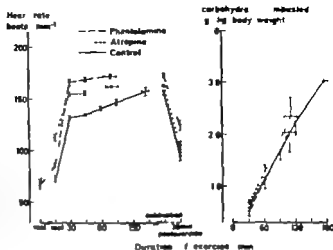


Fig. 4 Mean values (\pm S.E.) of heart rate and amount of carbohydrate ingested are plotted the entire time. When no samples had been drawn, they were administered.

exhaustion below preexercise (rest₀) values was significantly largest in control expts., and glucose concentrations were in control as well as in atropine expts. significantly lower than in phenolamine expts. 30 min postexercise glucose concentrations in phenolamine expts. did not differ significantly from preexercise concentrations, while glucose concentrations in control and atropine experiments still were below preexercise levels and still not significantly different from each other.

During exercise blood lactate concentrations were highest during α -adrenergic blockade (Fig. 5). 30 min postexercise lactate concentrations always had decreased below exercise levels, but were still above preexercise values. Alanine concentrations in serum were significantly increased after 30 min of exercise, but no longer at exhaustion, at which time the concentrations were significantly lowest in control expts. FFA and glycerol concentrations in serum gradually increased during exercise and were at identical times highest in phenolamine expts. (Fig. 3). At exhaustion the concentrations in control expts. did not differ significantly from concentrations in phenolamine expts., but were higher than concentrations in atropine expts. 30 min postexercise FFA and glycerol concentrations were still above preexercise levels (Fig. 3).

The exercise heart rate was increased by drugs and to the greatest extent during α -adrenergic blockade (Fig. 4). 30 min postexercise heart rate was still highest in phenolamine expts., in both most subjects were dizzy after exercise. Administration of drugs did not influence hematocrit values at rest. During exercise slight decrease in hematocrit (4% of preexercise value) took place only in phenolamine and atropine expts.

Discussion

Acute α -adrenergic blockade with phenolamine markedly increased norepinephrine concentrations during exercise (Fig. 2, 5). Similarly schizophrenics treated for months with chlorpromazine, an α -adrenergic blocking agent, had higher plasma norepinephrine con-

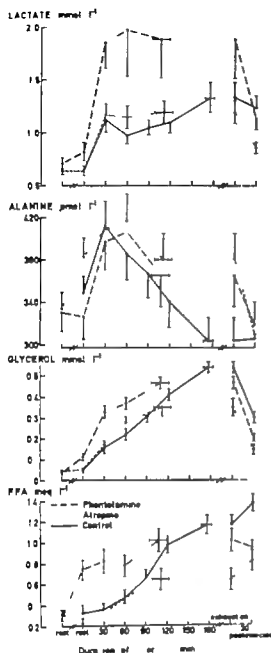


Fig. 3. Mean concentrations (\pm SE) of blood and of alanine, glycerol and free fatty serum obtained during rest and prolonged re-plotted at the work time. When rest-sam been drawn, drugs were administered.

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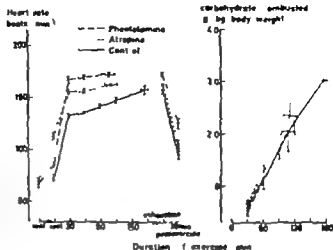


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Discussion

α -adrenergic blockade with phenolamine markedly increased norepinephrine concentrations during exercise (Fig. 2, 5). Similarly schizophrenics treated for months with doxapramine, an α -adrenergic blocking agent, had higher plasma norepinephrine con-

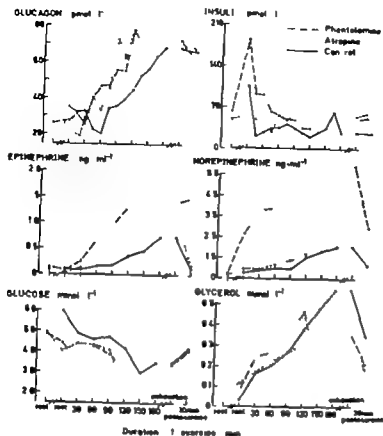


Fig. 5 The entire course of the plasma concentrations of catecholamines, pancreatic hormones, glucose and glycerol obtained during rest and prolonged exercise in one subject (HG). When rest, samples had been drawn, drugs were administered.

centrations during exercise than untreated subjects (Carlsson *et al.* 1968). These observations may be explained by increased norepinephrine overflow to the blood stream due partly to direct actions of α -adrenergic blocking agents at sympathetic nerve endings leading to an increased norepinephrine concentration within the synaptic gap upon a single nerve impulse (Langer 1974) and partly to increased sympathetic nervous activity. Increased sympathetic nervous activity may have been provoked in response to low arterial blood pressures during exercise after administration of α -adrenergic blocking drugs (Carlsson *et al.* 1968, Reinbeimer, Davidson and Albrink 1968). Furthermore, since it cannot be excluded that phentolamine reduces $\dot{V}_{O_2 \max}$, the relative load on the working muscles possibly was higher in phentolamine expts. than in control expts. even at the same level of oxygen uptake. A higher relative work intensity however cannot completely account for the high norepinephrine concentrations obtained during α -adrenergic blockade, since these concentrations are even higher than the concentrations obtained during treadmill running of maximal intensity (Galbo *et al.* 1975). Moreover as indicated by identical carbohydrate combustion rates (Fig. 4) and modest differences with respect to lactate concentrations (Fig. 3) and pulmonary ventilations (53.84 ± 3.95 (P) and 48.55 ± 3.84 (C) l (STPD) min⁻¹ mean and S.E., $n = 7$, $p < 0.02$) between phentolamine and control expts. the relative load on the working muscles did not differ much in these expts. (Asmussen 1965).

Also during exercise after administration of atropine, a drug that does not change $\dot{V}_{O_2 \max}$ (Ekblom *et al.* 1972), plasma norepinephrine concentrations were higher than in control

expts. (Fig. 2, 5). Acetylcholine released during vagal stimulation has been shown to reduce norepinephrine overflow into the coronary sinus through activation of muscarinic receptors (Langer 1974, Levy and Blattberg 1976). Furthermore, the vagal tone on the heart is partially but not completely withdrawn at a work load of 60% of maximal oxygen uptake (Ekblom *et al.* 1977). These findings agree with the supposition that abolition by atropine of an acetylcholine induced inhibition of norepinephrine release from cardiac postganglionic sympathetic terminals caused the higher plasma norepinephrine concentrations after atropine in the present expts. The lower epinephrine/norepinephrine ratio obtained at exhaustion in phentolamine expts. compared with atropine and control expts. (Fig. 2) may partly be explained by a reduced overflow of norepinephrine from synaptic gaps per nerve impulse during phentolamine infusion (Langer 1974). Since a decrease in plasma glucose concentrations has been shown selectively to enhance the epinephrine response to prolonged exercise (Galbo *et al.* 1977) also the lower plasma glucose concentrations reached during exercise in atropine and control expts. (Fig. 1) may to some extent account for the higher epinephrine/norepinephrine ratio obtained in these expts. compared with phentolamine expts.

Phentolamine does not influence the secretion of pancreatic hormones from the isolated, perfused pancreas (Iversen 1973). Nevertheless in the present study plasma insulin concentrations are increased at rest by phentolamine (Fig. 1, 5). The increments in insulin concentrations correlated significantly with the simultaneous increments in norepinephrine concentrations. These findings suggest that when B cell inhibiting α -receptors were blocked by phentolamine at rest, stimulation of pancreatic B cell enhancing β -receptors by increased transmitter concentrations predominated (Gench, Charles and Grodsky 1976, Harvey *et al.* 1974, Luyckx and Lefebvre 1974). The finding that during exercise insulin concentrations also decreased in phentolamine expts. (Fig. 1, 5) in which the increase in catecholamine concentrations was marked, probably was due to the fact that phentolamine is a competitive blocking agent and to the existence of more α - than β -adrenergic receptors on pancreatic B cells (Clark *et al.* 1976). However as previously found in rats (Harvey *et al.* 1974, Luyckx and Lefebvre 1974) throughout exercise plasma insulin concentrations remained higher during adrenergic blockade than in control expts. (Fig. 1, 5). In rats the estimated rate of insulin action has been found to decrease during exercise, and this decrease could be abolished by adrenergic blockade (Brisson, Malahue Lagas and Malahue 1971). Thus a diminished insulin secretion caused by α -adrenergic activity probably accounts for the decreased plasma insulin concentrations found during prolonged moderate exercise in the postabsorptive state (Clark *et al.* 1976, 1977, Harvey *et al.* 1974, Luyckx and Lefebvre 1974), whereas it appears in the present study (Fig. 1) that the parasympathetic nervous system does not significantly influence the insulin response to this kind of exercise. Nor is a decrease in plasma insulin concentrations of major importance for the decrease in plasma insulin concentrations during moderate exercise since we have shown that maintenance of euglycemia by glucose infusion does not diminish the exercise induced decrease in insulin concentrations (Galbo *et al.* 1977).

Neither α -adrenergic blockade nor parasympathetic blockade abolished the glucagon response to exercise (Fig. 1, 5). Nor was the glucagon response enhanced in phentolamine expts. even though the stimulation of β -adrenergic receptors in these expts. probably was

Intensified by the particularly high catecholamine concentrations (Fig. 1). It might be argued that differences in plasma substrate concentrations possibly blurred differences in glucagon secretion (Gerich *et al* 1976). However at identical times in the different expts. plasma alanine concentrations were similar (Fig. 3), and we have recently shown that free fatty acids do not significantly influence the glucagon response to exercise (Galbo *et al* 1976). Furthermore in the first 60 min of exercise plasma concentrations of glucose as well as of glucagon were identical whether autonomic blockade was accomplished or not. During the last part of exercise the decline in glucose concentrations differed in the different experiments (Fig. 1) but as one would expect (Galbo *et al* 1976, 1977; Gerich *et al* 1976), the rates of decline of glucose concentrations were closely matched to the rates of increase in glucagon concentrations (Fig. 1). Thus in agreement with our previous study of glucagon and plasma catecholamines during β -receptor blockade (Galbo *et al* 1976) the present study could not demonstrate any significant modulating effect of α or β -receptors on the glucagon response to exercise in man. The applied drugs undoubtedly changed the cardiovascular dynamics (Fig. 4) (Ekblom *et al* 1972; Reinheimer, Davidson and Albrink 1968). This may have affected the elimination of insulin and glucagon hormones which are both predominantly cleared in the liver and kidney. However since the concentrations of insulin and glucagon did not change in parallel during the expts. and furthermore 30 min postexercise still were identical to concentrations at exhaustion, differences in elimination rates of pancreatic hormones caused by effects on the circulation of phentolamine and atropine are not likely to affect the interpretation of the results.

At identical times during exercise plasma concentrations of FFA and glycerol were highest in phentolamine expts. (Fig. 3). This finding indicates according to studies of the relationship between the plasma concentrations of these substances and their rates of production and disappearance at constant work loads (Shaw, Isselkutz and Isselkutz 1975) that lipolysis and muscular uptake of FFA was enhanced during α -adrenergic blockade. Conversely β -adrenergic blockade has been shown to inhibit lipolysis at rest and during exercise (Day 1975; Galbo *et al* 1976, 1977). Although muscular uptake of FFA probably was increased in phentolamine expts., as judged from analysis of expired air the rate of fat combustion was not increased. However it has been shown that free fatty acids taken up by skeletal muscle are not necessarily oxidized immediately (Zierler 1976). Furthermore in phentolamine expts., as indicated by higher blood lactate levels (Fig. 3) an increased muscular glycogenolysis elicited by increased β -receptor stimulation (Day 1975; Galbo *et al* 1976) possibly exerted a relative inhibition of lipid oxidation. The finding that at exhaustion after different work times FFA and glycerol values were identical in spite of higher catecholamine concentrations in phentolamine than in control expts. may be explained by the fact that in control expts. lower plasma concentrations of insulin, which is an antilipolytic hormone, were maintained during a longer work time (Fig. 1-5). The present study was unable to unveil a parasympathetic inhibition of lipolysis (Colvillo *et al* 1964; Weiss and Maickel 1965). Thus FFA and glycerol concentrations in plasma were not increased during parasympathetic blockade with atropine (Fig. 3-5), a finding which could not be explained by differences in lipid combustion rate (Fig. 4) or in catecholamine and insulin levels between atropine and control expts. In conclusion the present study indicates that during prolonged exercise in man the secretion

insulin is depressed by stimulation of α -adrenergic receptors whereas the secretion of glucagon is not significantly influenced by adrenergic receptors. The stimulation of β -adrenergic receptors enhances lipolysis but neither lipolysis nor the secretion of pancreatic enzymes are influenced by cholinergic activity during exercise.

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K⁺-Permeability of the Blood-Brain Barrier, Investigated by Aid of a K⁺-Sensitive Microelectrode

By

ANKER JON HANSEN, HENRIK LUND-ANDERSEN and CHRISTIAN CRONE

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Abstract

HANSEN A. J. H. LUND-ANDERSEN and C. CRONE. *K⁺-permeability of the blood-brain barrier investigated by aid of a K⁺-sensitive microelectrode* Acta physiol. scand. 1977 438-445

The K⁺-permeability of the blood-brain barrier at the capillary level was estimated from decrease of brain extracellular K⁺-concentration in response to an isotonic bolus containing KCl injected in carotid artery. A very low permeability appeared from the fact that the extracellular K⁺-concentration measured by aid of K⁺-sensitive microelectrodes—remained unchanged during the passage of the bolus. An upper limit for the blood-brain barrier K⁺-permeability in the rat was estimated to be 10^{-10} cm

The extracellular K⁺-concentration in the brain is kept approximately constant despite considerable and long lasting changes of the plasma K⁺-concentration (Bradbury and Leman 1967, Cohen *et al.* 1968). However during spreading depression, anoxia or several large transient changes of the extracellular K⁺-concentration occur (Vyskočil *et al.* 1971, Prince *et al.* 1973, Hansen 1977). The K⁺-permeability of the blood-brain barrier is important for these phenomena since brain extracellular K⁺-concentration might be influenced by the passage of K⁺ between brain and blood across the barrier. Also an exact knowledge of the K⁺-permeability of the blood-brain barrier would be important for a thorough analysis of the DC potential between brain extracellular fluid and blood.

So far two methodological approaches, the tissue uptake technique (Katzman and Leman 1953, Bradbury and Kleeman 1967) and the indicator diffusion technique (Crone and Thompson 1970) have been applied to study the K⁺-permeability of the blood-brain barrier. These studies have revealed a very low permeability. However a detailed quantitative interpretation of the data obtained with these two methods in terms of blood-brain barrier permeability at the capillary level is difficult for slowly penetrating solutes since transport occurs across many interphases besides the blood brain barrier: the arachnoid membrane, the choroid plexus and structures lacking a blood-brain barrier. The indicator diffusion technique has the particular complication that the test solute (⁸⁶K) and the reference substance may be separated intravascularly by Taylor's effect (Lassen and Crone 1970).

The complication is particularly disturbing when dealing with substances which permeate slowly through the blood-brain barrier because of the difficulty of distinguishing between intravascular separation of test solute and reference substance and a small separation due to a minute transcapillary permeation of the test solute.

Demon and Welch (1971) approached the problem of a quantitative assessment of the K⁺-permeability of the blood-brain barrier by fitting a mathematical model to brain and CSF space data. However ingenious, this approach remains indirect, and the permeability data are necessarily encumbered with some uncertainty since several parameters of the model simulating the entire system are not yet known with great accuracy.

The introduction of K⁺-sensitive microelectrodes (Walker 1971) permits a more direct determination of capillary permeability to K⁺ which we have employed in the present study.

A preliminary report was presented at the XVth meeting of the Scandinavian Physiological Society (Hansen and Lund-Andersen 1976).

Methods

Experimental procedures: The experiments were performed on 27 adult male Sprague-Dawley rats weighing 300-350 g. The animals were anesthetized with pentobarbital administered i.p. (50 mg/kg) and cannulae were placed in the trachea. One catheter (pp 23) was retrogradely guided to the arch of the aorta through the abdominal aorta. Another catheter (pp 10) was placed in the external carotid artery with the tip situated near its carotid bifurcation (Murray and Phelps 1972). The head of the rat was mounted in a head holder and the catheters cleared. The sagittal sinus was exposed through a burr hole in the skull 3-4 mm to the left of the coronal suture. The dura and the connective tissues overlying the sinus were carefully dissected so that the blood stream was visible. Through another burr hole the parietal cortex was partially exposed and small incision was made in the dura without any damage to the pia. Two small Ag-AgCl electrodes were placed diametrically opposite around the burr hole for recording of the EEG.

The animals were paralyzed with galliumcitrate sodium and artificially ventilated at settings to maintain normal blood gases and pH. The blood pressure was monitored via the catheter in the aorta. The rectal temperature was kept constant at 37°C by servocontrolled heating system.

K⁺-sensitive microelectrodes: The potassium concentration in the extracellular fluid and in the sagittal sinus was measured with K⁺-sensitive microelectrodes. The manufacturing and use of double-barrelled glass microelectrodes has previously been described (Hansen 1977). The tip diameter was about 1 µm and the impedance of the barrel containing potassium ion exchanger (Corning 477317) was 10⁸-10⁹ MΩ.

One electrode was placed in the sagittal sinus. Another electrode was positioned in the parietal cortex through the slit in the dura. The tip of the electrode was located 200-700 µm below the surface by means of a micromanipulator. In few cases the electrode was placed in the extracellular fluid of neck muscle or the meninges. The animals were grounded via glass tubes filled with 150 mM KCl embedded in the neck muscle. The experimental set-up is shown schematically in Fig. 1. The voltage from the electrodes was recorded by a high resistance DC-amplifier with input resistance of 10⁹ MΩ. For each electrode the potential difference between the two barrels indicated the K⁺-concentration, the relation between K⁺-concentration and potential being approximately logarithmic with a slope of 40-50 mV for 10-fold change in K⁺-concentration. The voltage between the reference barrel of the cortex electrode and the common ground measured the DC-potential between brain extracellular fluid and ground. All potentials were recorded on a multi-channel UV-recorder (Southern Instruments).

The K⁺-electrodes were found to be very sensitive to galliumcitrate sodium as they are sensitive to tetraethylammonium chloride (Nieber and Lux 1973), this complicated the administration of galliumcitrate. Thus, 15-20 min had to elapse between the initial administration of galliumcitrate and placement of the electrode. When additional galliumcitrate was needed the sinus electrode was removed and replaced after 10 min passed. When this procedure was followed the sinus electrode measured the plasma K⁺-concentration independently of the plasma K⁺ of about 5 mM (Fig. 2). The electrode in the brain was unaffected by galliumcitrate administration indicating that this substance does not pass the blood-brain barrier.

K⁺-Permeability of the Blood Brain Barrier, Investigated by Aid of a K⁺-Sensitive Microelectrode

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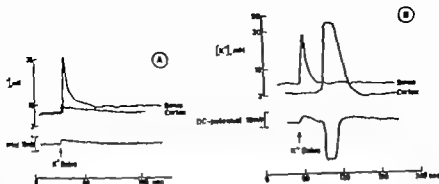
Abstract

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The K⁺ permeability of the blood-brain barrier at the capillary level was estimated from determinations of brain extracellular K⁺-concentration response to an isotonic bolus containing KCl injected into carotid artery. A very low permeability appeared from the fact that the extracellular K⁺-concentration measured by aid of K⁺-sensitive microelectrodes—remained unchanged during the passage of the bolus. An upper limit for the blood-brain barrier K⁺ permeability in the rat was estimated to be $2.8 \cdot 10^{-4}$ cm

The extracellular K⁺-concentration in the brain is kept approximately constant despite considerable and long lasting changes of the plasma K⁺-concentration (Bradbury and Kleeman 1967, Cohen *et al.* 1968). However, during spreading depression, anoxia or seizure, large transient changes of the extracellular K⁺-concentration occur (Vyskočil *et al.* 1971, Prince *et al.* 1973, Hansen 1977). The K⁺-permeability of the blood-brain barrier is important for these phenomena since brain extracellular K⁺-concentration might be influenced by the passage of K⁺ between brain and blood across the barrier. Also, an exact knowledge of the K⁺ permeability of the blood-brain barrier would be important for a thorough analysis of the DC potential between brain extracellular fluid and blood.

So far, two methodological approaches, the tissue uptake technique (Katzman and Leidman 1953, Bradbury and Kleeman 1967) and the indicator diffusion technique (Crone and Thompson 1970) have been applied to study the K⁺ permeability of the blood-brain barrier. These studies have revealed a very low permeability. However, a detailed quantitative interpretation of the data obtained with these two methods in terms of blood-brain barrier permeability at the capillary level is difficult for slowly penetrating solutes since transport occurs across many interphases besides the blood-brain barrier: the arachnoid membrane, the choroid plexus and structures lacking a blood-brain barrier. The indicator diffusion technique has the particular complication that the test solute (⁴²K) and the reference substance may be separated intravascularly by Taylor's effect (Lassen and Crone 1970).



The course of the K^+ -concentration in the sagittal sinus, the K^+ -concentration in brain extracellular fluid and the DC-potential between brain extracellular fluid and ground. The figures show some atypical responses to KCl injections. The left part (A) illustrates a situation where a minor increase of brain extracellular K^+ -concentration occurred. The right part (B) illustrates a situation where the extracellular concentration remained constant whilst the bolus passed the brain, but 35 s later the K^+ -concentration and the DC-potential changed as during a spreading depression.

passed through the brain. The K^+ -concentration in the extracellular fluid, however, was less than 0.1 mM . This picture was seen in response to 77 injections in 27 animals. In the DC-potential between sinus and ground remained constant whilst the DC-potential on brain extracellular fluid and ground showed a transient deflection (brain positive). It is consistently found that the DC-potential change lasted longer than the passage of the bolus. The sign of the potential shift is compatible with a KCl diffusion potential with permeability Cl^- -permeability and in accordance with data given by Cameron (1970) by Medes and Mines (1975). However, the fact that the potential change was maintained after the bolus had passed the brain seems to indicate a more complex genesis which is not understood at present.

When the solution contained $NaCl$ instead of KCl no changes were observed in K^+ and Cl^- values.

In a few animals atypical pictures appeared as illustrated in Fig. 3 A and 3 B. Fig. 3 A was a situation when a slight increase in the K^+ -concentration was registered in the brain (sinus), in Fig. 3 B a spreading depression reaction was elicited after the bolus passage (sinus). The changes in extracellular K^+ -concentration and DC-potential during this phenomenon are well known (Vyskočil *et al.* 1972).

Other tissues

In order to examine the response in organs with a significant capillary K^+ -permeability, experiments were performed on striated muscle and mesentery. Fig. 4 A shows the K^+ -concentration in the extracellular fluid of a neck muscle and in the sagittal sinus in response to a bolus injection through the aortic catheter. Contrary to what was seen in the brain the bolus passage was clearly reflected in the extracellular fluid of the muscle. The experiments were somewhat disturbed by muscle contraction due to the potassium-depolarization and were only successful in 2 out of 12 animals. In the mesentery however it was easy to demonstrate a large increase in the extracellular K^+ -concentration when a bolus was injected through a

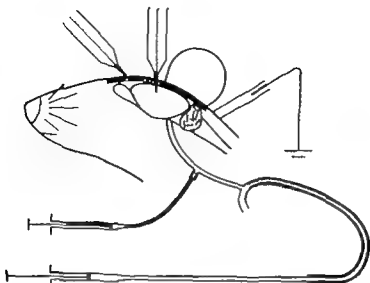


Fig. 1. Schematic presentation of the experimental set-up. Double-barrelled K^+ -sensitive electrodes are positioned in the sinus and in the extracellular fluid of the brain. One catheter is in the proximal part of the carotid artery while another is led from the aorta to the arch of the aorta.

Injection through the catheters. Boluses of 75–200 μ l KCl -solutions in phosphate buffer (pH 7.4) were injected through the catheters over 1–2 s. In the aortic catheter 1 M KCl solutions were used. 60–150 mM solutions were used in the carotid catheter. $NaCl$ -solutions of similar tonicity served as controls. Results obtained when the KCl -solutions were injected either through the carotid or the aortic catheters were indistinguishable and the data will accordingly be described and discussed together.

Results

Brain

Fig. 2 shows the K^+ -concentration in the sagittal sinus and in brain extracellular fluid together with the DC-potential between sinus and ground or brain and ground. The figure illustrates the typical response when a KCl -bolus was injected either through the carotid or the aortic catheter. It appears that the K^+ -concentration in the sagittal sinus varied in a typical organ outflow curve after intra-arterial injection, indicating that the bolus

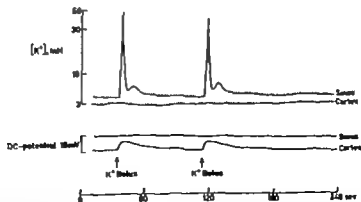
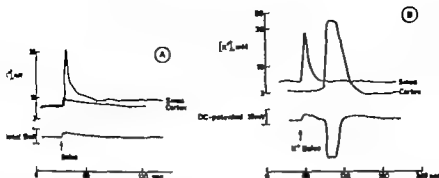


Fig. 2. Time course of the K^+ -concentration in the sagittal sinus, the K^+ -concentration in brain extracellular fluid, the DC-potential between reference barrel of the sinus electrode and ground and the DC-potential between brain extracellular fluid and ground. 75 μ l of a 150 mM KCl solution was injected over 1–2 s per bolus through the carotid catheter. A small recirculation peak appears in the sinus. Note that the change in the brain-ground DC-potential only slowly declined after the bolus had passed the brain. When the potential was normalized after 80 s the injection of KCl was repeated.



The course of the K⁺-concentration in the sagittal sinus, the K⁺-concentration in brain extracellular fluid and the DC-potential between brain extracellular fluid and ground. The figures show some atypical upon KCl injections. The left part (A) illustrates situation where minor decrease of brain extracellular K⁺-concentration occurred. The right part (B) illustrates situation where the extracellular K⁺-concentration remained constant whilst the bolus passed the brain, but 35 min later the K⁺-concentration and the DC-potential changed as during spreading depression.

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When the solution contained NaCl instead of KCl no changes were observed in K⁺ and DC-potential. In a few animals atypical pictures appeared as illustrated in Fig. 3 A and 3 B. Fig. 3 A shows a situation when a slight increase in the K⁺-concentration was registered in the brain (bolus), in Fig. 3 B a spreading depression reaction was elicited after the bolus passage (bolus). The changes in extracellular K⁺-concentration and DC-potential during this reaction are well known (Vyakochil *et al.* 1972).

Experiments in muscle

In order to examine the response in organs with a significant capillary K⁺-permeability experiments were performed on striated muscle and mesentery. Fig. 4 A shows the K⁺-concentration in the extracellular fluid of a neck muscle and in the sagittal sinus in response to a bolus injected through the aortic catheter. Contrary to what was seen in the brain the bolus passage was clearly reflected in the extracellular fluid of the muscle. The experiments were somewhat disturbed by muscle contraction due to the potassium-depolarization and were only successful in 2 out of 12 animals. In the mesentery however it was easy to demonstrate an increase in the extracellular K⁺-concentration when a bolus was injected through a

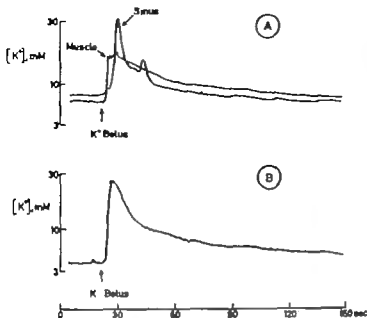


Fig. 4 (A) Time course of the K concentration in the sagittal sinus and in the extracellular fluid of neck muscle when a KCl-bolus was injected through the catheter with the tip placed in the aortic arch. (B). Time course of the K concentration in the extracellular fluid of the mesentery when a KCl-bolus was injected through catheter with the tip placed near the coeliac trunk.

catheter in the abdominal aorta (Fig. 4 B). Intracellular location of the electrodes can be excluded since the DC potential between reference barrel and ground was close to 0 mV. These expts. clearly show that in tissues with a relatively high K permeability of the capillaries changes of the plasma concentration are rapidly and significantly transmitted to the extracellular fluid.

Discussion

The constancy of the extracellular K-concentration when a KCl containing bolus passes the brain leaves little doubt that the blood brain barrier is close to being impermeable to potassium. The sources of error in the experimental design are few. The electrode tip views the events in a minute region somewhere between the capillary and the cellular structures of the neuropil. Since the mean intercapillary distance is about 30–40 μm (Diemer 1968) the passage of potassium into a local brain region from a capillary should rapidly be monitored by the electrode, as illustrated in muscle and mesentery. Other sources of error include artefacts of the local capillary perfusion by the electrode. This is, however, unlikely since the extracellular K-concentration around the electrode tip remained low throughout an experiment indicating sufficient supply of blood to the neighbouring cells. Another complication would be arteriolar smooth muscle contraction due to the depolarizing effect of the high potassium in the bolus. The rapid passage of the bolus indicates, however, that there was no flow interference. This possibly signifies that the permeability of the endothelium in arterioles is similarly low.

Even though uncontrollable experimental errors seem to be small we cannot conclude that the blood-brain barrier has zero permeability to potassium since there is a lower limit to the electrode response. Thus, instead of postulating zero permeability we have calculated an upper limit for the permeability using the following reasoning.

Determination of a permeability coefficient of the blood-brain barrier to potassium

as to spontaneous small potential fluctuations observed with the cortex-electrode changes of less than 0.5 mV are regarded as insignificant. A 0.5 mV change corresponds in the 3 mM sodium region to 0.1 mM change in K^+ -concentration. Thus, the apparent constancy of x brain K^+ -concentration during a bolus passage through the vascular system only safely indicates that the K^+ -concentration in the extracellular fluid did increase less than 0.1 mM. This means that in a hemisphere weighing 1 g with an extracellular space of 15% less than $1.5 \cdot 10^{-4}$ mmol K^+ has passed the blood-brain barrier (disregarding uptake of K^+ in brain cells). In expts. where KCl was injected through the external carotid artery the amount of potassium supplied to the hemisphere on the side of injection can be calculated from the time-concentration curve recorded in the sagittal sinus as $F(1 - Hct) \cdot A$, where F is blood flow, Hct is haematocrit— $F(1 - Hct)$ is thus the plasma flow—and A is the integral of the time-concentration curve (mmol ml⁻¹ s). This last value is multiplied by 2, because the concentration in the blood perfusing the hemisphere on the injected side is approximately twice as high as after the dilution in the sinus with a similar amount of blood from the non-injected side. The area under the potassium curve in the sagittal sinus is 110 μ mol ml⁻¹ s in the example shown in Fig. 2. With a Hct of 0.45 and a blood flow of 1/60 ml g⁻¹ s⁻¹—the normal value for rat cortex under well-ventilated conditions—these figures yield a maximal extraction, E , of $0.015(1/60 - 0.55 \cdot 2/110) \cdot 100 = 0.74\%$. However since the Hct on the injected side is lowered due to the addition of the red cell free bolus the value of E is further reduced. If the Hct were lowered to 0.40 unilaterally E would be 0.68%. If it were 0.35 E would be 0.63%. In the subsequent calculations we chose a Hct of 0.40. Our value for K^+ permeability for this reason alone would be an upper estimate.

There are previous reports of potassium extraction during a single capillary passage in the brain. Crone and Thompson (1970) reported a ^{42}K -extraction of 2.2%, a figure which is clearly too high. The explanation of this falsely high figure is intravascular separation of potassium from Evans Blue Dye which was used as reference solute and transendothelial loss in the choroid plexus and blood-brain barrier free areas. Lassen *et al.* (1971) using ^{86}Rb as tracer arrived at an extraction of 1.4%, a figure which is most likely too high, again due to the same complications, although they carefully corrected for Taylor-effects. Available correction methods in cases with very low extraction, are however of limited accuracy.

With knowledge of the extraction a potassium permeability can be calculated from $P = (F/3)(1 - Hct) \ln(1/E)$ where S is capillary surface area (240 cm²) in 1 g of brain (Crone, 1965). Under the proviso that the potassium permeability is independent of concentration P is $2.8 \cdot 10^{-6}$ cm sec⁻¹. This value is two orders of magnitude smaller than that for muscle capillaries (Renkin and Shochan 1970), illustrating the extremely low permeability of the blood-brain barrier.

Denson and Welch (1971) approached the quantitative assessment of the potassium permeability by modelling whole brain data from Bradbury and Davson (1965) and from Bradbury and Kleeman (1967) and taking into account the extra supply of ^{42}K from the choroid plexus. Using the same capillary areas as we have used their model gave a P_K of $5.6 \cdot 10^{-6}$ cm sec⁻¹ for K^+ across the blood-brain barrier. In view of the heterogeneous nature of the

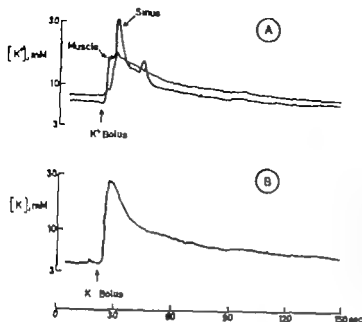


Fig. 4 (A) Time course of K^+ concentration in the supratentorial and in the extracellular fluid of the neck muscle when a KCl bolus was injected through the ear with the tip placed in the arch. (B) Time course of K^+ concentration in the extracellular fluid of the mesentery when a bolus was injected through a catheter with the tip placed near coeliac trunk.

catheter in the abdominal aorta (Fig. 4 B). Intracellular location of the electrodes can be excluded since the DC-potential between reference barrel and ground was close to 0. These experiments clearly show that in tissues with a relatively high K^+ permeability of the capillaries changes of the plasma concentration are rapidly and significantly transmitted to extracellular fluid.

Discussion

The constancy of the extracellular K^+ -concentration when a KCl containing bolus passes the brain leaves little doubt that the blood-brain barrier is close to being impermeable to potassium. The sources of error in the experimental design are few. The electrode tip is in the events in a minute region somewhere between the capillary and the cellular structure of the neuropil. Since the mean intercapillary distance is about 30–40 μm (Diemer 1968) passage of potassium into a local brain region from a capillary should rapidly be monitored by the electrode, as illustrated in muscle and mesentery. Other sources of error include an occlusion of the local capillary perfusion by the electrode. This is, however, unlikely since the extracellular K^+ -concentration around the electrode tip remained low throughout an experiment, indicating sufficient supply of blood to the neighbouring cells. Another complication would be arteriolar smooth muscle contraction due to the depolarizing effect of the high potassium in the bolus. The rapid passage of the bolus indicates, however, that there was no flow interference. This possibly signifies that the permeability of the endothelium in arterioles is similarly low.

Even though uncontrollable experimental errors seem to be small we cannot conclude that the blood-brain barrier has zero permeability to potassium since there is a lower limit to the electrode response. Thus, instead of postulating zero permeability we have calculated an upper limit for the permeability using the following reasoning.

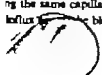
of a permeability coefficient of the blood-brain barrier to potassium

aneous small potential fluctuations observed with the cortex-electrode changes ± 0.5 mV are regarded as insignificant. A 0.5 mV change corresponds in the 3 mM agon to 0.1 mM change in K^+ -concentration. Thus, the apparent constancy of K^+ -concentration during a bolus passage through the vascular system only safely states that the K^+ -concentration in the extracellular fluid did increase less than 0.1 mM. In a hemisphere weighing 1 g with an extracellular space of 15% less than of K^+ has passed the blood-brain barrier disregarding uptake of K^+ in brain tissue where KCl was injected through the external carotid artery the amount of K^+ applied to the hemisphere on the side of injection can be calculated from the extraction curve recorded in the sagittal sinus as $F(1 - Hct) \Delta A$, where F is blood flow, Hct hematocrit— $F(1 - Hct)$ is thus the plasma flow—and A is the integral of the extraction curve ($\mu\text{mol ml}^{-1} \text{s}$). This last value is multiplied by 2, because the K^+ in the blood perfusing the hemisphere on the injected side is approximately halved after the dilution in the sinus with a similar amount of blood from the non-injected side. The area under the potassium curve in the sagittal sinus is $110 \mu\text{mol ml}^{-1} \text{s}$ as shown in Fig. 2. With a Hct of 0.45 and a blood flow of $1/60 \text{ ml g}^{-1} \text{s}^{-1}$ —the value for rat cortex under well-ventilated conditions—these figures yield a maximal K^+ extraction of $0.015/(1/60 \cdot 0.55 \cdot 2 \cdot 110) \cdot 100 = 0.74\%$. However since the Hct on the injected side is lowered due to the addition of the red cell free bolus the value of E is further lowered. If the Hct were lowered to 0.40 unilaterally E would be 0.68%. If it were 0.35 E would be 0.63%. In the subsequent calculations we chose a Hct of 0.40. Our value for K^+ extraction for this reason alone would be an upper estimate.

In previous reports of potassium extraction during single capillary passage in the rat brain, Lassen and Thompson (1970) reported ^{42}K -extraction of 2.2%, a figure which is high. The explanation of this falsely high figure is intravascular separation of ^{42}K from Evans Blue Dye which was used as reference solute, and transendothelial fenestrations and blood-brain barrier free areas. Lassen *et al* (1971) using ^{22}Na reported an extraction of 1.4%, a figure which is most likely too high, again due to complications, although they carefully corrected for Taylor-effects. Available methods in cases with very low extraction, are however of limited accuracy.

From the knowledge of the extraction a potassium permeability can be calculated from $P_K = (1 - Hct) \ln(1 - E) / \delta$ where δ is capillary surface area (240 cm^2) in 1 g of brain tissue. Under the proviso that the potassium permeability is independent of concentration P_K was $2.8 \cdot 10^{-11} \text{ cm sec}^{-1}$. This value is two orders of magnitude smaller than the permeability of the choroid plexus (Rankin and Sheehan 1970), illustrating the extremely low permeability of the blood-brain barrier.

Andersson and Welch (1971) approached the quantitative assessment of the potassium permeability by modelling whole brain data from Bradbury and Davson (1965) and from Bradbury and Welch (1967) and taking into account the extra supply of ^{42}K from the choroid plexus. Their model gave a P_K of $5.6 \cdot 10^{-11} \text{ cm sec}^{-1}$. In view of the heterogeneous nature of the



entire blood-brain-CSF system our results lend a valid support to the Davson and Welch data and a slight revision in the downward direction. Our upper limit for the potassium permeability brings this figure closer to the figures for sodium and chloride permeability given by Davson and Welch in the same paper $0.9 \cdot 10^{-4} \text{ cm s}^{-1}$.

The figure for P_K of $1.4 \cdot 10^{-4} \text{ cm s}^{-1}$ reported by Rapoport (1976) results from a calculation of Davson and Welch's figure for P_K of $5.6 \cdot 10^{-4} \text{ cm s}^{-1}$. Rapoport derived $t_{1/2}$ of 68 min from this figure by assuming that the entire brain could be treated as a single compartment with a fractional volume of distribution for K^+ of 0.8. This is hardly correct since the apparent volume of distribution for K^+ is much larger than 0.8 due to the high intracellular K^+ -concentration. Using a half-time of 68 min Rapoport derives a P_K for the passage from plasma to extracellular fluid of brain (fractional volume of 0.2) of $1.4 \cdot 10^{-4} \text{ cm s}^{-1}$ ($0.2/0.8 \cdot 5.6 \cdot 10^{-4}$). This method of recalculating Davson and Welch's data is erroneous for the reason mentioned and Davson & Welch's figure cannot be changed unless the model is reformulated.

Our value of P_K for the blood-brain barrier of $2.8 \cdot 10^{-4} \text{ cm s}^{-1}$ (per cm capillary surface) represents the as yet lowest experimentally determined permeability. The 0.5 mV detection limit, the capillary area of $240 \text{ cm}^2/\text{g}$ for cortex and the hematocrit of 0.40 are likely to cause overestimation of P_K and it must, therefore, be emphasized that although this P_K is very low the true P_K may well be even lower. It is, therefore, not yet possible to know with certainty whether P_K and P_{Cl} are different or similar. Such knowledge would have more than academic interest in view of its importance for the understanding of the genesis of the DC-potential across the blood-brain barrier.

Expert technical assistance from Mrs Marianne A. Sørensen is gratefully acknowledged.

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Sustained Water Diuresis in Anesthetized Dogs Antidiuresis in Response to Intravenous and Bilateral Intracarotid Infusion of Hyper-Osmolar Solutions of Sodium Chloride

By

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Abstract

BIE, P. Sustained water diuresis in anesthetized dogs: Antidiuresis in response to intravenous and bilateral intracarotid infusion of hyper-osmolar solutions of sodium chloride. *physiol. scand* 1977 101 446-457.

The function of the suggested hypothalamic osmoreceptors was re-examined in dogs during light chloroform anesthesia. The body weight of the dogs was increased to—and maintained at—102.0% of the initial by 1 h infusion of a hypo-osmolar solution of glucose and urea. During the water diuresis renal free clearance (C_{H_2O}) remained approximately constant for more than 3 h during which infusion of physiological amounts of vasopressin (3 or 10 μ U/(kg b.wt. min)) was associated with a dose-dependent, reversible decrease in C_{H_2O} . Infusion of hyper-osmolar sodium chloride solution (90 μ mol/(kg b.wt. min)) for 10 min was performed either through a central venous catheter or through needles placed in both common carotid arteries. After an initial increase in C_{H_2O} these infusions elicited antidiuresis (negative C_{H_2O}) and led to increases in the rates of excretion of sodium (100-fold) and potassium (3-fold). Left atrial pressure and renal clearance of inulin and PAH did not change significantly. The renal effects of NaCl infusion were independent of the mode of infusion. It is concluded that the results are inconsistent with the hypothesis that the common carotid arteries include an osmoreceptor which is capable of changing—within seconds—the rate of secretion of vasopressin, although they support the view that the renal excretion of water is strongly influenced by a receptor which is sensitive to osmolality or concentration of sodium in plasma. In addition it appeared that an increase of a few per cent in the concentration of sodium in plasma is associated with a substantial increase in the rate of excretion of sodium without measurable change in central venous pressure or in glomerular filtration rate.

It is generally accepted that the rate of excretion of water is largely determined by the concentration of vasopressin in blood plasma. This concentration however is known to be influenced by many factors of which only the osmolality and the volume of some extracellular compartment are supposed to be elements of the regulation mechanisms which maintain the composition and volume of the extracellular fluid within physiological limits. Changes in blood or extracellular fluid volume can be detected by stretch receptors placed within suitable structures e.g. the atrial wall. In their critical review Goetz, Bond and Blomhorn (1975) concluded, however, "that the left atrial volume-receptor hypothesis ad

to our current understanding of the regulation of the extracellular fluid balance" and posited that some measure of concentration as the variable to which the rate of secretion of vasopressin is adjusted under physiological circumstances. The physiological knowledge of this regulatory mechanism—called an "osmoreceptor"—seems indisputable (Schoen and Athar 1976). In contrast to Verney's interpretation of his results (1947) recent evidence (Ble 1976 a) indicates that the receptor is not situated within the carotid arteries. In the latter experiments, no antidiuresis was elicited by infusion into the carotid arteries of hyper-osmolar sodium chloride solutions in dosages ≥ 10 g/l. Administration neither restored plasma osmolality to normal nor elicited diuresis. The present experiments were designed to evaluate the validity of these results covering the following question: Does antidiuresis develop when the concentration of sodium in plasma of the anesthetized water-diuretic dog approaches normal values as a result of administration of hyper-osmolar saline? and—if so—is the antidiuretic response to iv infusion different from the response to bilateral intracarotid administration of hyper-osmolar sodium chloride solution at the same rate?

Methods

Male beagle puppies weighing approximately 10.8 kg (range 9.5–12.2 kg) were used. They were on a commercial dog diet and regular measurements of the daily excretion of sodium and potassium were of 41 ± 9.1 and 3.5 ± 0.1 mmol/kg b.wt., respectively (mean \pm S.E. = S.D.). Before the present series surgical dissection of the carotid arteries (Michez 1972) was performed in order to facilitate cannula puncture. Each animal was used for a number of experiments with intervals of at least 2 weeks. General anesthesia. Prior to experiments food was withheld for 18 h. The dog had free access to water. Atropine (40 μ g/kg b.wt.) was given 1/2 h before induction of anesthesia. A sterile through-the-needle catheter was introduced into a lateral cephalic vein and after collection of blood sample the dog was anesthetized with chloralose (60–70 mg/kg b.wt.). Throughout the experiment supplementary doses (usually 20 mg/kg b.wt.) were given. The chloralose was purified as described earlier (Ble 1976 a) dissolved (10 g/l) in a solution of glucose (40 mM) and urea (25 mM). This solution of glucose and urea is referred to as GU-solution. Small amounts of pentobarbital (5 to 10 mg/kg b.wt.) proved to be effective in preventing excitation during induction and was given in all experiments. Following intubation a second through-the-needle catheter was placed in or near the right atrium after dissection of the right external jugular vein. Appropriate priming doses of 14 C-urea and 3 H-p-azoximycin and (NEN Chemicals, Dreisbachsdamm, W. Germany) were then injected and an infusion of these tracers was started. 1 series of series (see below) puncture of both carotid arteries was performed as described earlier (Ble 1976 a). After insertion of a Pezzy catheter into the urinary bladder the dog was placed in the experimental set-up of which a diagram is shown in Fig. 1. This includes a control animal cell—electrodes—pump A—animal/food) capable of controlling the body weight of the animal within 0.1% of the desired value (Ble 1976 a). Zero adjustment of the control current of the servo motor and recording of the central venous pressure was performed between 18.00 and 19.10. At 19.10 collection of urine and the infusion of GU-solution was started (pump B, Fig. 1). 20 ml/kg b.wt. was infused during 60 min. From Fig. 1 can be seen that the function of pump B did not influence the control animal which was activated only by decrease in the total load (by evaporation and decrease in loading and period of infusion of GU-solution (0–60 min)) the experiments took 4 h, the test solution consisting of 0.9% NaCl was infused during the third hour (120–180 min corresponding to 00.10–01.10 p.m.). Urine which could not be performed according to the schedule were discarded. All experiments were really similar except for the composition, the volume, and the mode of administration of the infusate used by pump D (Fig. 1). Five series of experiments were performed. Control experiments. In these no test solution was infused and clearances of urea and PAH were measured. Pumps C and D (Fig. 1) were not in use.

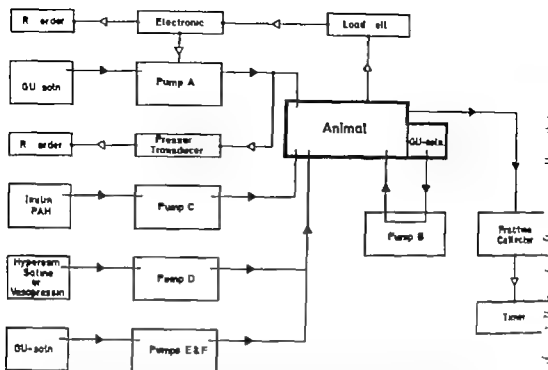


Fig. 1 Diagram of the experimental set-up. Filled arrows indicate routes of fluid flow. Open arrows indicate directions of actions which were electrical except for the gravitational input to the load cell and the input to the pressure transducer. The weight of the dog and a reservoir containing GU-solution (the weight of components of the double-contoured bag) was registered by the load cell and sustained by the animal, load cell, "electronics", and pump A. For the sake of simplicity the function of the timer and the temperature controlling systems have been excluded. The timer stopped the pump(s) infusing through the central venous catheter and simultaneously activated the recorder for a period of 40 s every time the fraction collector made a step. Le every 10 min. Pumps E and F were used in series only.

Series b. I. Infusion (0.2 ml/min for 60 min) of vasopressin ($5 \mu\text{U}/(\text{kg b.wt. min})$) dissolved in GU solution. Clearances of inulin and PAH were not measured.

Series c. I. Infusion (0.2 ml/min for 60 min) of vasopressin ($10 \mu\text{U}/(\text{kg b.wt. min})$) dissolved in GU solution.

Series d. I. v. Infusion (0.4 ml/min for 60 min) of hyper-osmolar sodium chloride solution ($90 \text{ mOsm}/(\text{kg b.wt. min})$). The concentration of NaCl was approximately 2.2 M.

Series e. Bilateral intracarotid infusion of hyper-osmolar sodium chloride solution similar to that given in series d, 0.2 ml/min was given into each artery. Before and after this infusion the cannulae were kept open by infusion of GU-solution (approximately 0.1 ml/min per artery, pumps E and F in Fig. 1).

Central venous pressure was measured every 10 min by a strain gauge transducer (Model P23BB, Statham Laboratories, Hato Rey, Puerto Rico) and an UV recorder (Model 3006, S.E. Laboratories, Felcham, England). The oscillations reflecting the heart beats were eliminated by an electronic filter which allowed the deviations caused by the respiratory activity to be seen. The maximal value during expiration was taken as the central venous pressure.

During anesthesia blood samples (3 ml) were obtained through the central venous catheter. Samples indicated in Fig. 2, sampling was each time followed by injection of an equivalent volume of dextran (Macrodex-saline, Pharmacia, Uppsala). Coagulation was prevented by dry heparin and centrifugation. Separation of plasma was started 1–2 min after sampling.

The initial injection of chloralose and all infusions through the central venous catheter were performed through sterile filters (Millex® 0.22 μm , Millipore Corp., Bedford, Mass.). The rectal temperature of the conscious animal was measured in the morning and throughout the experiment. This temperature was kept close to its normal value ($\pm 0.2^\circ\text{C}$) by heating of both the operating table and the fluids delivered by pumps A and B.

Preparation. The USP Potassium Chloride Reference Standard was prepared and stored as recommended by Krimm (1947). The extract was eluted with GU-solution immediately before use. The rates of infusion were equivalent to $13 \cdot 10^{-10}$ and $25 \cdot 10^{-10}$ mol arginine-vasopressin/(kg b.wt. min).

Anal. and methods. Hematocrit was determined in quadruplicate immediately after sampling by centrifugation for 5 min at 20 000 g using micro-hematocrit capillaries. Osmolality was determined by an osmometer (Advanced Model 31 LASE) as described earlier (Baz 1976 a). On the day of the expt. the concentrations of Na^+ and K^+ in samples of plasma and urine were measured by means of a flame photometer (M. M. Instrumentation Laboratory Inc., Lexington, Mass.) using lithium as internal standard. When plasma was analyzed the apparatus was calibrated by use of the viscosity-adjusted standard solution provided by the manufacturer. Dilution by roller pump has been described as potential source of methodical error (Vader and Vink 1975). The rates of excretion of standard solutions, dog plasma, and water are assessed by accurate densitometry (Kratky Leopold and Stabinger 1969) and repeated weighing. The value for plasma and standard solution showed no significant difference (both were about 1% lower than for water obtained for water). Thus it was assumed that the results were uninfluenced by the use of the roller pump belonging to the flame photometer.

Samples of plasma and urine are deep-frozen until the concentrations of ^{14}C and ^3H are determined in a liquid scintillation counter (Liquid Scintillation System, Mark II, Nuclear-Chicago) using PCS⁹ (Pierce-Chicago) as scintillation fluid. The amounts of ^{14}C -inulin and ^3H PAH infused into the animals are adjusted so as to produce a ratio between ^3H and ^{14}C of about 10:1. In separate expts. the concentration of water in plasma during water diuresis was found to be 93.1% (v/v). Therefore, the clearance of inulin was multiplied by 0.931 in order to obtain glomerular filtration rate. The rate at which Na^+ was cleared into the renal tubules was calculated as glomerular filtration rate times the concentration of Na^+ in plasma. The concentration of glucose in plasma was determined in several expts. by the method described by Sauer, Roy and Wadlinger (1970), the values after hydration with GU-solution (mean $7.03 \text{ mM} \pm 0.8 \text{ E.}$ 0.1 mM , 30) were not different from those measured in plasma obtained before hydration (mean $7.43 \text{ mM} \pm 0.3 \text{ E.}$ 0.1 mM , 20). The urine was examined by use of N-Labids (Ames Co. Skids Pogue, England) before and during the experiments. Proteins, glucose, ketone bodies, and bacteria were never detected in the urine but traces of hemoglobin were occasionally present.

Recovery. The recovery after chloralose anesthesia was rapid and uneventful, no sequelae could be detected by clinical inspection.

Results

Results are given as mean values derived from series of 6 consecutive expts. Within each series consistent responses were obtained. The results obtained from analysis of samples of arterial blood are shown in Fig. 2, the values which were obtained in series b and c were indistinguishable from those obtained in series a. The infusion of GU-solution from 10 to 11.10 lowered the osmolality of plasma by 3-4% and produced a slightly larger decrease in the concentration of Na^+ . The mean concentration of K^+ exhibited slight variations. Apparently the values obtained in the morning in series d and e are higher than those of series a-c. The reason for this is not clear. Separate expts. in conscious dogs showed that application of venous stasis (cuff pressure of 70-90 mmHg for 5 min) was not followed by any changes in the concentration of potassium in plasma, therefore, the observed discrepancy cannot be ascribed to different degrees of venous stasis during blood sampling. From Fig. 2 it can be seen that once the hydration was established the concentrations of Na^+ and K^+ and the osmolality did not change throughout the control experiments. Infusions of hyper-osmolar NaCl solution (series d and e) caused an increase in plasma osmolality and in the concentration of sodium in plasma as well as a decrease in the concentration of K^+ and a delay of the steady increase in hematocrit which was seen in series a. The direction and magnitude of these responses are compatible with the assumption

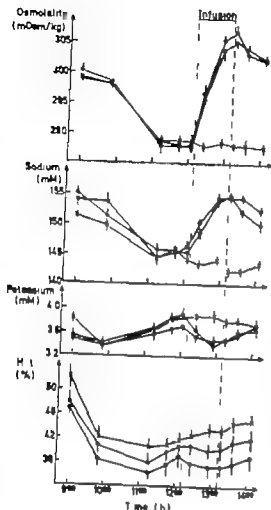


Fig. 2 Hematocrit, concentrations of Na^+ and plasma, and plasma osmolality during different of expts. Control expts (○) in which no test is was infused. Intra-venous (○) and bilateral carotid (●) infusions of hyper-osmolar sodium chloride solutions, $90 \mu\text{mol}/(\text{kg b wt. min})$ and $45 (\text{kg b wt. artery min})$, respectively. The animal anesthetized at 09.00–09.30 a.m. and infusion solution was performed during the period indicated. Values are mean \pm S.E., $n=6$.

that infusion of the hyper-osmolar sodium chloride solution caused a shift of water from intracellular to the extracellular fluid space.

Water excretion and osmolar clearance

Diuresis and osmolar clearance in the course of the series of experiments are shown in Fig. 3. Free water clearance is equal to the vertical distance between the two graphs shown in Fig. 3. In part of the figure, it is positive when diuresis is greater than osmolar clearance. The infusion of vasopressin caused a decrease in diuresis and in free water clearance (Fig. 3 b and c) and these effects were apparently dependent on the rate at which vasopressin was infused.

From Fig. 3 d and 4 it is seen that the immediate response to i.v. infusion of hyper-osmolar NaCl solution was biphasic. During the first half of the infusion the osmolar clearance was unchanged whereas the diuresis (Fig. 3 d) and the free water clearance (Fig. 4) increased. During the last part of the infusion period the osmolar clearance showed a substantial increase while the diuresis and the free water clearance declined. Negative free water clearance (Fig. 4 and intersection of graphs in Fig. 3 d and 3 e) appeared approximately 10 min after termination of the infusion.

From Fig. 3 d, 3 e and 4 it seems justified to conclude that the renal response to infusion

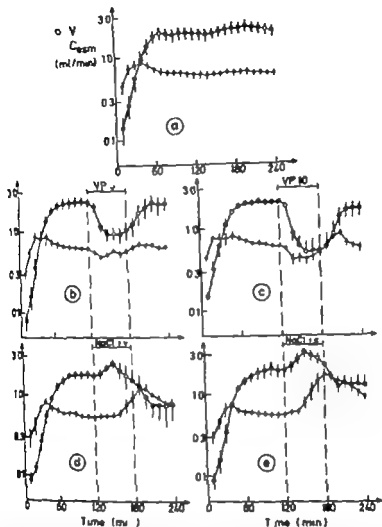


Fig. 3. Diuresis (O) and osmolar clearance (●) as function of time during 5 series of expts: control (a), infusion at constant rate in period 0-60 min followed by sample attainment in period 60-240 min, (b) infusion of vasopressin at rate of $5 \mu\text{U}/(\text{kg} \cdot \text{min})$, infusion of vasopressin at rate of $10 \mu\text{U}/(\text{kg} \cdot \text{min})$, (c) intravenous infusion of hyper-osmolar sodium chloride solution at rate of $90 \text{ mmol}/(\text{kg} \cdot \text{min})$, (d) intravenous infusion of hyper-osmolar sodium chloride solution at rate of $45 \text{ mmol}/(\text{kg} \cdot \text{min})$, (e) intravenous infusion of hyper-osmolar sodium chloride solution at rate of $45 \text{ mmol}/(\text{kg} \cdot \text{min})$. Periods of infusion of vasopressin or sodium chloride are indicated by dashed lines. Time 0 corresponds to 10.10 min. Note the logarithmic spacing of the vertical scale. Values are mean \pm S.E., $n=6$.

into the common carotid arteries of a strongly hyper-osmolar sodium chloride solution was not significantly different from the response to i.v. infusion. The effects of NaCl infusion differed from those of vasopressin administration by (i) an initial increase in free water clearance, (ii) appearance—within the period of infusion—of an increase in osmolar clearance, and concurrent with the latter (iii) a considerable increase in the rate of excretion of sodium.

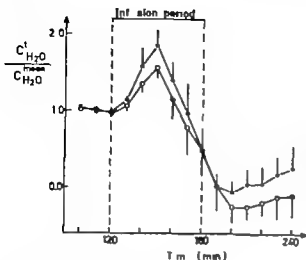


Fig. 4 Changes in free water clearance (O) and intracarotid (●) infusion hyper-osmolar sodium chloride solution at a rate of $90 \mu\text{mol}/(\text{kg b.w.t. min})$. In each of the free water clearance to each of the 14 periods of collection ($C_{H_2O}^1$) was divided the mean of the three clearances preceding infusion. *i.e.* $C_{H_2O}^1 = (C_{H_2O}^0 + C_{H_2O}^1 + C_{H_2O}^2)/3$. Time 0 corresponds to 10.10 m. The results are mean \pm S.E., $n=6$.

The rate of excretion of sodium

The rates of excretion of sodium and potassium during the series of expts. are shown in Fig. 5. panel *a* shows that the course of these rates in the control series was similar to that observed earlier (Bie 1976 b, 1976 c) *i.e.* an apparent increase during hydration followed a decrease in the rate of excretion of sodium whereas the rates of excretion of potassium throughout the expts. remained at an elevated level when compared to prehydration values. This pattern was not changed by administration of vasopressin (Fig. 5 b and 5 c). During the infusion of hyper-osmolar sodium chloride solution the rate of excretion of sodium increased from about $1.0 \mu\text{mol}/\text{min}$ to $100\text{--}150 \mu\text{mol}/\text{min}$ (Fig. 5 d and 5 e). During the infusions no change could be measured in either the clearance of p-aminohippuric acid or in glomerular filtration rate, therefore, fractional sodium excretion increased concomitantly with the absolute rate of excretion *i.e.* the rate of excretion of sodium increased from less than $3 \cdot 10^{-4}$ to $0.02\text{--}0.04$ times the rate of filtration. The rate of excretion of potassium showed a modest increase and the ratio between the concentrations of sodium and potassium in urine changed from approximately 0.1 before infusion to a maximum of 4–5 just after the infusion.

The results thus show that the rates of excretion of Na⁺ and K⁺ increased 100-fold and 3-fold, respectively when plasma osmolality and sodium concentration was normalized by infusion of hyper-osmolar sodium chloride solution following after sodium-free volume expansion and that apparently identical alterations occurred irrespective of the mode of administration.

Central venous pressure

The results of the measurements of the central venous pressure are shown in Fig. 6. The values obtained in series *b* and *c* (infusions of vasopressin) were not significantly different from—or were lower than—those obtained in control expts. From Fig. 6 it is seen that the hydration procedure caused a lasting increase in central venous pressure of about 1.0 cm H₂O. It also appears that the i.v. infusion of hyper-osmolar sodium chloride solution was not associated with values of central venous pressure different from 1.

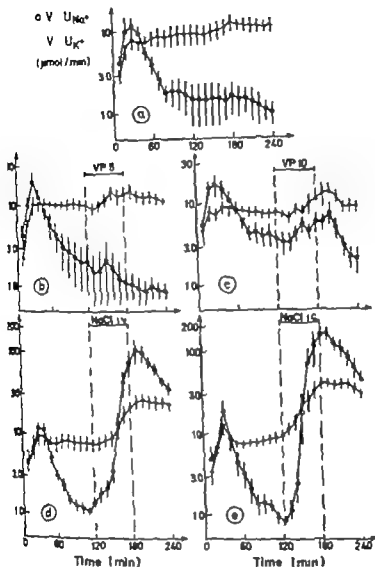


Fig. 3. Rates of excretion of sodium (O) and potassium (e) as function of time during 5 series of expts.

experiments. Intracerebral infusions of hyper-osmolar saline was associated with a mean central venous pressure which were approximately 1.0 cm of water above stained in the control series.

Discussion

These expts. were designed in order to re-examine the osmoreceptor hypothesis (1947). According to this hypothesis the covariation of the osmolality of the blood

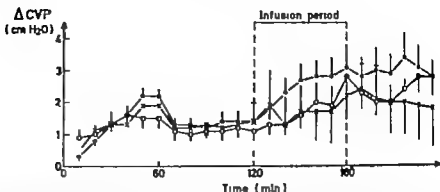


Fig. 6. Changes in central venous pressure as function of time in control expts. (○) and in 2 series of expts. in which hyper-osmolar NaCl solution was infused i.v. (○) or i. to the carotid arteries (●). Hydration was performed in the period 0–60 min. Pressure = 0 corresponds to the value obtained at time = –5 min, time 0 at 10.10 a.m. Results are mean \pm S.E., $n = 6$.

and the rate of secretion of vasopressin is mediated by an "osmoreceptor" situated within the area supplied by the common carotid arteries. The results of the present expts. support the view that diuresis and the rate of excretion of "free water" may be controlled by an "osmoreceptor" but they are inconsistent with the hypothesis with regard to the location of the receptor. This statement rests upon several assumptions concerning the concentration of vasopressin in plasma during the expts. and the osmolality to which the anterior part of the hypothalamus was exposed during the intracarotid infusions. The possible influence of the anesthetic and the volume of the hydration fluid on the rate of secretion of vasopressin has been dealt with earlier (Bie 1976 a, 1976 b), in addition, the validity of the conclusions drawn from the present results may be questioned (i) if the infusions of hyper-osmolar sodium chloride solutions were not performed during a water diuresis (defined as a condition characterized by a low rate of secretion of vasopressin), (ii) if the renal antidiuretic response to the infusion of the NaCl solutions was elicited exclusively by factors other than an increase in the concentration of vasopressin in plasma, and (iii) if the regional hyperosmolality created by the bilateral infusions into the common carotid arteries did not include the anterior hypothalamic area in which the "osmoreceptors" are said to be localized (Jewell and Verney 1957 Woods, Bard and Bleker 1966).

(i) In the present expts. solutions containing NaCl or vasopressin were infused into animals under circumstances apparently fulfilling all criteria of a water diuresis. The diuresis measured approximately 0.2 ml/(kg b wt. min), urine osmolality was between 75 and 120 mOsm/kg of water, the rate of excretion of sodium was low and the urine did not contain abnormal amounts of glucose. The concentration of urea in the GU-solution was roughly equivalent to the concentration in the urine during the experiments and there is no reason to expect, therefore, that the infusion of the GU-solution caused an increase in the concentration of urea in plasma. It thus appears that the diuresis increased as a result of changes different from those eliciting an osmotic diuresis. The sensitivity to exogenous vasopressin strengthens the impression of a water diuresis. Graded antidiuretic responses were obtained by infusions of 55 and 110 μ U/min to dogs weighing approximately 11 kg. From expts. on conscious dogs with diabetes insipidus weighing 7–12 kg Shannon (1947) concluded that graded antidiuresis

obtained by infusion of 0.001–0.005 units per hour corresponding to 17–83 μ U/min. *in vivo* it was concluded that dogs in chloralose anaesthesia excrete a water load as rapid as conscious animals (Theobald 1934). Thus it seems justified to assume that the condition in which vasopressin or hyper-osmolar solutions were infused was that of a water load elicited by a considerable decrease in the rate of secretion of vasopressin.

(i) It is apparent that the renal response to infusion of the NaCl solutions included details which most probably cannot be due to an increase in the rate of secretion of vasopressin. However, under the present circumstances during which the clearance of *p*-aminohippuric acid and the glomerular filtration rate did not show any decrease it seems highly improbable that the renal response—which included a decrease in mean free water clearance from 1.2 \pm 4 ml/min to values below zero—could take place without a considerable increase in the rate of secretion of vasopressin.

(ii) Calculations of the intensity of the osmotic stimulus to which the anterior hypothalamus is exposed during intracarotid hyper-osmolar stimulation require information concerning the degree of mixing of the infusate with carotid blood and the blood flow through the common carotid arteries. When slow intracarotid infusions are used in studies of osmotic stimulation it is generally assumed that complete mixing occurs within the common carotid artery (Bac 1976 a, Eriksson, Fernández and Olsson 1971, Verney 1947, Ikeda, Clarke and Minton 1956). Consistent responses in series of consecutive experiments in different animals support the validity of this assumption. It seems unlikely that large parts of the infusate in all experiments would by-pass the origins of the internal carotid artery and the ramus anastomotic of the maxillary arteries through which—at least in the dog—the circulation arteriosus is supplied (Gillilan 1976). It therefore seems reasonable to assume—as Verney (1947) did—an approximate increase in the osmolality of the blood rising the hypothalamus from the rate of infusion of NaCl and an estimate of the blood flow through the common carotid arteries.

It thus appears that none of the said three reservations apply to the present studies. The effect of the intracarotid infusion on the osmolality of the carotid blood depends on the blood flow and the rate of infusion. Assuming a flow through a common carotid artery of 50 ml (Fig. 6 a \pm 1 mm) it can be calculated (Bac 1976 a) that the intracarotid infusions increased the osmolality by some 12 mOsm/kg of water. *I.e.* in series *c* the osmolality of the blood rising the carotid arteries exceeded the values shown in Fig. 2 (upper panel) by this amount throughout the infusions. By considering the slopes of these graphs during infusion it is seen that at the very beginning of the intracarotid infusions the osmolality of the carotid blood increased to prehydration values which during *iv* infusion were not obtained until some 25 min later. It also appears that at the end of the *iv* and the intracarotid infusions the carotid osmolality was approximately 305 and 320 mOsm/kg, respectively. Despite these relatively large differences the antidiuretic responses in the two series of experiments were indistinguishable.

Previous work (Bac 1976 a) did not include results which supported any osmoreceptor theory; therefore, from these negative experiments it was concluded that the validity of the hypothesis of Verney appeared questionable. The present results are in accordance with the view that the rate of secretion of vasopressin is accurately controlled by a receptor mechanism

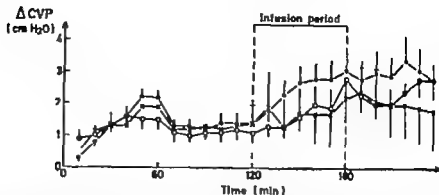


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It thus appears that none of the said three reservations apply to the present studies. The effect of the intracarotid infusion on the osmolality of the carotid blood depends on the blood flow and the rate of infusion. Assuming a flow through a common carotid artery of 100 ml/kg b.w.t. min it can be calculated (Bac 1976 a) that the intracarotid infusions increased the osmolality by some 12 mOsm/kg of water *i.e.* in series the osmolality of the blood reaching the carotid arteries exceeded the values shown in Fig. 2 (upper panel) by this amount throughout the infusions. By considering the slopes of these graphs during infusion it is seen that at the very beginning of the intracarotid infusions the osmolality of the carotid blood increased to prehydration values which during *i.v.* infusion were not obtained until 25 min later. It also appears that at the end of the *i.v.* and the intracarotid infusions carotid osmolality was approximately 305 and 320 mOsm/kg, respectively. Despite these relatively large differences the antidiuretic responses in the two series of experiments were indistinguishable.

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The rate of excretion of sodium

The present work shows—in accordance with previous results (Bie 1976b)—that hypo-osmolar volume expansion is associated with a decrease in the rate of excretion of sodium. The present expts. with vasopressin infusions show that this hormone may cause a decrease in diuresis and an increase in urine osmolality without a concomitant increase in the rate of excretion of sodium. It has been shown repeatedly that vasopressin is capable of enhancing this variable (*e.g.* Buckalew and Diamond 1976, Humphreys, Friedler and Earley 1970), however the phenomenon has been demonstrated only by infusion of vasopressin at rates which were larger than any reasonable estimate of the maximal rate of secretion in the neurohypophysis. It is uncertain whether naturally occurring concentrations of vasopressin may induce an increase in the rate of excretion of sodium.

The changes in absolute as well as fractional sodium excretion during infusion of hyper-osmolar sodium chloride solutions seem massive and the chain of events between the infusions and the increase in the rate of excretion of sodium deserves further investigation.

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over but the cellular transmembrane potential changes are more sensitive in revealing the effects of shock at the cellular level and precede significant interstitial electrolyte changes.

Various types of hypoperfusion shock conditions will result in a heterogeneous distribution of the microvascular flow (see e.g. Nicoll and Frayser 1967) and severely interfere with cellular metabolic function (Baze *et al.* 1974). Assuming that transmembrane potential is sensitive in revealing the cellular metabolic consequences of hypoxia in single skeletal muscle fibres, then the extent of heterogeneity in distribution of nutritive flow should be demonstrable from such transmembrane potential measurements.

In the present study a model with exteriorization of the intestine, resulting in severe shock (Bergentz *et al.* 1969), was used. The cellular membrane potential changes in the progress of shock was registered. Comparison was made to the transmembrane potential reaction to complete ischemia.

Material and Methods

Mixed dogs (ca. 17), weighing 20–28 kg, were used. After atropine 0.5–0.5 mg and diazepam 5 mg i.v., anesthesia was induced with ketamine 2.5 mg/kg b.w. The dogs were intubated after muscular relaxation with pancuronium bromide and ventilated with air to keep P_aCO_2 of 4.0–4.5 kPa. Asepsis was maintained throughout the experiment by the use of slow ketamine infusion and additional doses of pancuronium bromide.

One of the carotid arteries was cannulated for continuous registration of blood pressure, for repeated blood analysis as respiratory control, and for hematocrit determinations. The preparation was allowed to stabilize for approximately 1 h before the experiment.

The following groups of experimental animals were studied.

Group 1 Control animals, subjected to anesthesia, insertion of catheters and sampling procedures. Sham laparotomy consisting of laparotomy without exteriorization of the intestine was performed.

Group 2 Shock was induced by exteriorization of the small intestine (in the addition of slight strangulation as previously described (Bergentz *et al.* 1969). After 3 h the intestine was replaced and the abdominal wall closed. The dogs were studied for an additional period of 2–3 h.

Group 3 Torusquectomy of one of the hind limbs was produced by the application of a narrow blood pressure cuff at 300 mmHg after removal of the blood with an Esmarch bandage. The torusquectomy was kept in place for 3 h and then released. A 3-h post-torusquectomy time period was studied. The same sham laparotomy as Group 1 was also performed.

Modified Lang-Garrard microelectrodes were used for the transmembrane potential registrations (Lang and Garrard 1949; Lennquist *et al.* 1969). The microelectrodes were drawn from borosilicate capillary tubing having diameter 1 (5–1.95 mm) using mechanical puller. The tip of the electrode was gently broken under microscope in order to give sharp tip of 1–2 μ m. The microelectrodes were filled with 1 M KCl. Electrodes with tip resistance of 4–10 megohms were used. The tip potentials were usually 1–2 mV. A small (outer diameter 2 mm) Ag-AgCl electrode the tip of which was inserted subcutaneously served as a reference electrode. The microelectrode was connected to high input impedance electrometer amplifier and penmanometric recorder (LKB 6500).

A small incision of approximately 1 cm² was made down to the fascia of the gracilis muscle. The surface was covered with sterile oil and spread out as gently performed in the fascia to expose the muscle. With the aid of microscope the microelectrode was advanced through the oil until contact was made with the muscle fiber. This was considered the zero point. The microelectrode was slowly advanced through the muscle, and the transmembrane potential was registered for the penetrated muscle cells. The transmembrane potential of the most superficial cells was always low. On passing through successive layers of muscle cells, there was gradual increase in transmembrane potential until, at depth of about 1–2 mm, fairly stable plateau was reached. Registrations from these superficial cells were not included in the material, as they probably represented injured cells (Cunningham *et al.* 1971a). A penetration was considered successful

Transmembrane Potential Measurements as an Indicator of Heterogeneous Distribution of Nutritive Blood Flow in Skeletal Muscle during Shock

By

HENGO HALJAMÄE, EVA JENNISCHE and ALF MEDEGÅRD

Received 9 June 1977

Abstract

HALJAMÄE, H. E. JENNISCHE and A. MEDEGÅRD *Transmembrane potential measurements as an indicator of heterogenous distribution of nutritive blood flow in skeletal muscle during shock*. Acta physiol. scand. 1977 101 458-464.

The heterogeneity of nutritive skeletal muscle blood flow during shock was studied in terms of cell transmembrane potential variations. Shock was induced in dogs by exteriorization of the small intestine for 3 h, whereafter the intestine was replaced and the dogs were studied for another 3-4 h. Transmembrane potentials of single skeletal muscle cells were recorded by the use of modified Lug-Gerard microelectrode. The mean resting transmembrane potential in control animals was $-90.4 \text{ mV} \pm 0.7$. The variation in transmembrane potential between adjacent fibers was small. During shock there was a significant decrease in the mean transmembrane potential and at the same time there was an increasing variation of up to 29 mV in the values between adjacent fibers. To exclude metabolic differences between red and white fibers as the main reason for this increasing heterogeneity of transmembrane potentials of adjacent fibers, complete tourniquet ischemia was also studied. The total tissue ischemia resulted in a marked reduction of the transmembrane potential in all cells with only small differences between adjacent fibers. After release of the tourniquet a marked spread of resting membrane potentials, with variations of 17-25 mV between adjacent fibers occurred. This variation was similar to that observed during shock. It is concluded that repeated transmembrane potential registrations may reveal the variable state of adjacent cells subjected to a heterogeneous nutritive blood flow in shock.

Key words. Skeletal muscle, hypoperfusion shock, transmembrane potentials, nutritive blood flow

Transmembrane potential measurements have been used as a possible method for direct monitoring of the pathophysiological changes occurring at the cellular level in skeletal muscle in shock (Campion *et al* 1969, Cunningham *et al* 1971b, Shires *et al* 1972, Trunkey *et al* 1973), ischemia (Arango *et al* 1976), and in severely ill patients (Cunningham *et al* 1971a). The changes in transmembrane potential seem mainly due to "hidden" local changes in the electrolyte distribution across the cell membrane during shock, as shown by direct sampling and analyses of the electrolyte content of interstitial fluid (Hagberg *et al* 1968, Haljamäe 1970a, 1970b) and of single cells (Haljamäe 1970c). Cunningham *et al* (1971b) found, how-

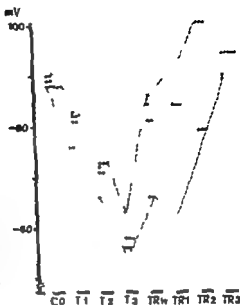


Fig. 3 Ten consecutive transmembrane potential registrations from adjacent cells before (CO), after 1, 2 and 3 h of complete tourniquet occlusion (T), and after 1, 2 and 3 h following release of the tourniquet (TR), are exemplified for one animal. Dotted lines indicate single measurements.

Transmembrane potential registrations from control animals during prolonged anesthesia for up to 6 h is exemplified in Fig. 1. Ten consecutive registrations at each time period are plotted. There were no significant changes in mean transmembrane potential during the control period. The variation in transmembrane potential between adjacent cells was very small, since all cells measured maintained a transmembrane potential close to -90 mV throughout the control period.

During shock there was a progressive decrease in mean transmembrane potential, and in addition there was an increasing variation in transmembrane potential between adjacent cells. This is exemplified in Fig. 2.

In Fig. 3 the effect of complete ischemia on the transmembrane potential is exemplified. Prolonged ischemia resulted in a significant decrease in the mean transmembrane potential, the decrease was more pronounced than that seen after corresponding time period of shock. The values of adjacent muscle fibers were at a similar level, i.e. the variation between adjacent cells was small and comparable to that during control conditions. After the release of the tourniquet there was a continuous increase in mean transmembrane potential. At the same time, however, there was a markedly increased variation in transmembrane potential between adjacent cells. After about 3 h transmembrane potentials returned to preischemic values, with small variation between adjacent fibers.

In Table I the data from 6 dogs in each experimental group are presented. Each individual value in the Table, represents the mean of 10 consecutive successful registrations from adjacent cells \pm S.E.M. at one penetration into the tissue. As can be seen from the Table, early (1-2 h) as well as late (4-5 h) shock resulted in a significantly ($p < 0.01$) decreased transmembrane potential. Tourniquet ischemia caused similar ($p < 0.01$) decrease in mean transmembrane potential.

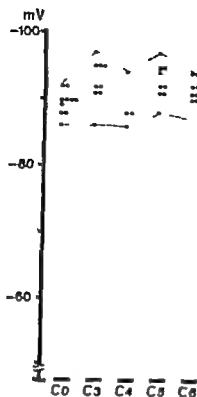


Fig. 1

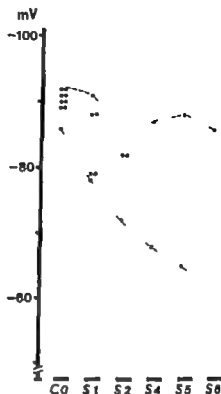


Fig. 2

Fig. 1. Separate transmembrane potential values of ten consecutive registrations from adjacent cells various times in one control experiment. C0-C6 indicate hours after start of the experiment. Dotted lines indicate range limits.

Fig. 2. Ten consecutive transmembrane potential registrations from adjacent cells exemplified before (C) and after 1 to 6 h of intestinal exteriorization shock (S1-S6) in one animal. Dotted lines indicate range limits.

If it was possible to keep the tip of the electrode within the cell for some seconds, and if the registration during this time remained at a rather stable niveau. Measurements were made at regular intervals and every time a fresh muscle area was exposed. Repeated registrations were made with several electrodes.

Wilcoxon's two-sample rank test (the Mann-Whitney test) was used for the statistical analysis.

Results

The intestinal exteriorization with slight stasis (Group II) resulted in a progressive decrease in blood pressure. The initial mean value was 141 mmHg , S.E. ± 5 . After 1 h a reduction to 112 ± 6 , after 2 h to 96 ± 12 and after 3 h to $72 \pm 11 \text{ mmHg}$ was observed. The replacement of the intestine and closure of the abdomen resulted in a transient slight increase to $78 \pm 10 \text{ mmHg}$, followed by a progressive circulatory deterioration and a blood pressure of only $35 \pm 11 \text{ mmHg}$ 3 h later.

Dogs subjected to tourniquet ischemia (Group III) had stable pulse and blood pressure except a slight transient decrease in blood pressure at the release of the tourniquet.

In control animals (Group I) pulse and blood pressure were mainly unchanged during the 6 to 7 h studied.

TABLE 1 Transmembrane potential registrations from skeletal muscle fibers during initial and 3 control conditions during early (1-2 h) and late (4-5 h) intestinal exteriorization shock 1-2 h of complete tourniquet ischemia and 1-2 h after release of the tourniquet. Each individual value represents the mean of ten consecutive successful registrations from adjacent cells \pm at one penetration into the tissue. 6 animals were studied in each group.

Initial values	Controls 2-3 h	Early shock 1-2 h	Late shock 4-5 h	Tourniquet 1-2 h	After release 1-2 h
-mV \pm S.D.	-mV \pm S.D.	-mV \pm S.D.	-mV \pm S.D.	-mV \pm S.D.	-mV \pm S.D.
88.9 1	89.6 2	83.6 4	78.3 4	79.9 3	77.4 6
89.5 2	92.2 3	79.0 5	77.7 8	65.8	88.6 9
89.0 3	93.7 3	85.6 6	70.3 7	79.2 3	73.6 6
89.7 2	93.2 3	84.0 7	72.0 7	70.7 3	82.7 6
91.9 3	84.1 1	82.8 6	75.1 8	63.6 3	88.6 6
93.1 3	88.1 2	81.3 5	65.1 9	66.7 3	89.2 7
Mean 90.4	90.2	82.7	73.1	71.0	83.4

$p < 0.01$ vs control values.

In each animal, however the individual values show that the shock induced decrease in mean transmembrane potential, is combined with an increased variation between adjacent cells. This variation increased throughout the shock period (*cf.* Fig. 2). If the individual S.D. values are taken as a measure of this variation, then significant ($p < 0.01$) differences were at hand between control animals and shock animals. Tourniquet ischemia, on the other hand, did not result in such an increased variation. After the release of the tourniquet increased variation ($p < 0.01$ vs controls) similar to that during shock occurred.

In control animals no changes in either the mean transmembrane potential or in variation between adjacent fibers were observed.

Discussion

The technique used for the transmembrane potential registrations was highly reproducible and the obtained control values of -88.9 – -93.1 mV for resting skeletal muscle potentials are in agreement with previous reports on mammalian skeletal muscle. The decrease in mean transmembrane potential in the course of the shock (*i.e.* exteriorization of the small intestine), was also similar to that previously reported for hemorrhagic shock in rats, dogs and primates (Camplon *et al.* 1969; Cunningham *et al.* 1971b; Shires *et al.* 1972; Truett *et al.* 1973; Arango *et al.* 1976).

There seems to be a direct correlation between the extent of cellular metabolic impairment and the mean cellular transmembrane potential reduction during shock (Jennische *et al.* 1977). It may therefore be assumed that such disturbances in cellular phosphagen and metabolite levels result in an interference with the cellular membrane electrolyte transport and increase in cellular sodium and loss of intracellular potassium (Haljamäe 1970b; Shires *et al.* 1977).

The increased variation in transmembrane potential between adjacent cells during shock may be considered as an indication of a marked difference in the metabolic deterioration

and adjacent cells and of an uneven distribution of nutritive blood flow in the muscle. It is known that the perfusion in the terminal vascular bed is rather heterogeneous (see also Fryer 1967). However during normal resting conditions vasomotion provides local nutritive flow to each cell. Direct intravital microscopic studies of the flow pattern in microvessels of skeletal muscle during shock has given direct evidence for a progressive loss of microvessels leading to an extensive "functional shunting" at the capillary level (Lund and Linder 1972). The studies of Appelgren (1972) of perfusion and diffusion in skeletal muscle by the use of clearance of ^{125}I for tissue perfusion and ^{131}I for tissue diffusion also indicate heterogeneity of nutritive flow during shock.

It has previously been reported that there is a difference in the resting transmembrane potentials between red and white muscles of the rat (Yocumura 1967) and Goodgold and Smith (1966) have suggested the existence of two families of muscle fibers in the human abdominal muscle, each having different transmembrane potential. No such grouping or subgroups of control resting membrane potentials could be seen in the muscle studied in present experiments. The tourniquet ischemia situation was used as a model to exclude the possibility of such a differential sensitivity of various groups of fibers to the hypoxia as an explanation for the observed spread of transmembrane potentials during shock. If such a differential sensitivity of various fibers were a main reason, then one would expect a similar spread of transmembrane potentials in the early phase of tissue anoxia after the release of the tourniquet. No such spread of the values was observed and the distribution only after the tourniquet application was similar to that during prolonged tourniquet ischemia. A diffusion may be assumed to equal out metabolic discrepancies between adjacent cells. After the release of the tourniquet, during the first 2 h of recirculation, there was, however, no spread of transmembrane potentials as observed during shock. This finding is well in agreement with the observations of Romanos *et al.* (1977) on the microcirculatory events during prolonged pressure-induced ischemia in the hamster cheek pouch. The release of the tourniquet after 3-4 h was reported to result in an initial low number of open microvessels, the number of which slowly increased to about 50% of controls after 2 h of recirculation. It is therefore concluded that transmembrane potential registrations may reveal functional differences between adjacent cells, which probably are caused by a heterogeneous distribution of nutritive blood flow to the cells.

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Electrophysiological Membrane Properties of the Frog Muscle Fibre: Effects of Detergents in the Triton Series

By

BO RYDQVIST

Received 9 June 1977

Abstract

Abstract. *Electrophysiological membrane properties of the frog muscle fibre. Effects of detergents in the Triton series.* Acta physiol. scand. 1977 101 465-475

The effect of the nonionic detergent Triton X 100 on the membrane properties of frog sartorius muscle fibre was studied using intracellular microelectrode technique. The effect of Triton X-100 was compared with that of Triton X-45 and Triton N-101. At $40 \mu\text{M}$ Triton X 100 had little effect on the specific membrane resistance (R_m), membrane capacitance (C_m) and the resting membrane potential (E_p) while the action potential (AP) was markedly reduced. At $160 \mu\text{M}$ AP was completely abolished and E_p was depolarized linearly with time. The effect on the maximum rate of rise (V_{max}) of the AP was dose-dependent and the apparent dissociation constant ($K_{\text{D app}}$) and K_{D} were found to be about $40 \mu\text{M}$. The Hill coefficient was 1.1, indicating deviation from first order reaction. The effect of Triton X 100 on E_p may be accounted for by reduction of the Na-K pumping activity. The effect of Triton X-100 on the AP is suggested to be due to perturbation of protein-systems either by drug-receptor interaction involving two or more sites, or specific binding of Triton X-100 to hydrophobic loci on the protein.

The anionic detergents have been extensively used in the solubilization of membranes (Dedrick and Simons 1975) and isolation of membrane-bound proteins, for example the cholinergic receptor protein (Miledi and Potter 1971; Meunier, Offen and Changaux 1972; Changaux *et al.* 1976). A number of nonionic detergents are known to cause a reduction of the contractile response of muscle to nerve stimulation (Soehring, Doss and Sta 1952). These detergents (e.g. alkyl-polyglycoethers) seem to have similar effects as local anaesthetics (Sta and Soehring 1952; Dittmann 1973) and produce anaesthesia of the cornea (Zipf and Dittmann 1964; Harris, Kahanowicz and Shlemmyo 1975). Furthermore, as shown by Branton, Devaux and Changaux (1975), Triton X 100 reversibly blocks the response of *Desmodium* electroplaque to carbamylcholine without affecting the resting membrane potential.

The nonionic detergents in the Triton series belong to a class of lipids called amphiphiles, that is, substances with a polar (hydrophilic) and an apolar (hydrophobic) part. Work on homologous alkyl-polyglycoethers (nonionic detergents similar to the Tritons) has yielded some insight into the relative importance of the hydrophobic and hydrophilic parts of

these molecules with regard to their blocking potency on the nerve-muscle-preparation (Zipf and Dittmann 1964, Dittmann 1973). However very little is still known about how detergents affect the membrane properties of muscle and nerve fibres.

The present experiments were carried out in order to obtain information about the influence of some Triton detergents on the active and passive electrophysiological properties of the frog muscle cell membrane. Classical drug-receptor kinetics have been used to describe some of the observed effects of Triton X 100.

Methods

The experiments were performed on the isolated frog sartorius muscle (*Rana temporaria*). The muscle is mounted in a bath with its deep surface upward and stretched to 110–120% of its resting length. The preparation was maintained at room temperature (about 23°C). The Ringer solution had the following composition: NaCl 115 mM, KCl 2.5 mM, CaCl_2 1.8 mM, Na_2HPO_4 2.15 mM and NaH_2PO_4 0.85 mM, pH 7.2 (Adrian 1956). The experimental procedure was the same as described in an earlier paper (Bohles and Rydqvist 1977).

Membrane potentials were recorded with glass capillary microelectrodes filled with 3 M KCl, resistance about 10 M Ω and tip potential of less than 5 mV. Microelectrodes used to inject current were filled with 2 M K-citrate, resistance about 10 M Ω . Care was taken only to impale surface fibres. In order to avoid penetrating the same fibre twice the electrodes were systematically moved across the muscle in one direction only. The current electrode was inserted about 25–50 μm from the voltage electrode and before the action potential was elicited the membrane was locally polarized to -90 mV in order to obtain uniform activation of all fibres.

Membrane potential changes were displayed on an oscilloscope coupled to the microelectrode through a high input impedance amplifier. In addition the resting membrane potential was monitored on a digital voltmeter. The current passed through the fibre was displayed on the oscilloscope as the voltage drop across the feedback resistor in an operational amplifier circuit used to hold the bath at virtual ground potential. The rate of rise of the action potential was obtained by differentiating the action potential electrically using a Miller differentiator and then displaying the output from the differentiator on the oscilloscope together with potential changes.

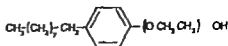
Membrane constants were obtained by rectangular pulse analysis for the case of an infinite cable (Hodgkin and Rushton 1946, Fatt and Katz 1951, Hodgkin and Huxley 1972, see also Bohles and Rydqvist 1977) and the specific membrane resistance (R_m) was calculated assuming the internal resistance of the fibre (R_i) to be 200 Ωcm (Fatt 1964).

Triton X-45 (TX-45), Triton X 100 (TX 100) and Triton N 101 (TN-101) were achieved from Rohm & Haas Co (Sweden) and used without further purification. TX-45 and TX 100 belong to a series of homologous polyoxyethylene *p*-4-octylphenols with the structural formula shown in Fig. 1 (Triton surface active agents, the nonionic octylphenoxyethanol (OPE) series, CS-40 I/cf Rohm & Haas Co, Philadelphia). TX-45 has a n -value between 4 and 5 and a molecular weight of 426. TX 100 has a n -value between 9 and 10 and a molecular weight of 628 (Rohm & Haas Surfactants, Handbook of Physical Properties CS-Ola, Rohm & Haas Co, Philadelphia). According to Simons *et al.* (1973) the mean value for TX 100 is 9.8. The hydrophilic/lipophilic balance number (HLB) is 10.4 for TX-45 and 13.5 for TX 100. None of these substances are homogeneous in their hydrophilic moiety being distributed according to Poisson (Schick 1967). Also the hydrocarbon part of the molecules is inhomogeneous to a small extent.

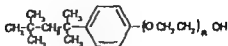
TN 101 is a polyoxyethylene nonylphenol (Fig. 1) with n between 9 and 10 and HLB number of 13.4 (Rohm & Haas Surfactants, Handbook of Physical Properties CS-Ola, Rohm & Haas Co, Philadelphia). The detergents were added to the Ringer solution to the actual concentrations.

Results

TX-45, TX 100 and TN 101 produced no gross morphological changes as observed in the dissecting microscope and no spontaneous contractions could be observed for the con-



TRITON N



TRITON X

Structural formula for the detergents in the X and Triton N series indicates the number isomeric units

actions used in the present study (*i.e.* 10–320 μM). The effects of TX 100 were primarily used and TX-45 and TN-101 were later applied at a few concentrations and the effects noted by these detergents compared to those of TX 100. The resting membrane properties studied using the resting membrane potential (E_m), the specific membrane resistance and the membrane capacitance (C_m). The maximum rate of rise (V_A) was used in analyses in the action potential (AP). This parameter was chosen as it is a good approximation for the sodium current (I_{Na}) underlying the muscle action potential.

Effects of Triton X 100

General effect of TX 100 on the muscle action potential is illustrated in Fig. 2 for three motoneurone fibres at different times. Record (A) shows the AP in normal Ringer solution. After 20 min in 160 μM TX 100 the muscle fibre AP is completely abolished (B) and the initial changes produced by the current pulses reflect the passive properties of the fibre only. After 20 min the muscle was returned to normal Ringer solution and about 10 min later an almost normal AP could be obtained (C). However the differentiated signal (per trace) clearly shows that the recovery was not complete. The maximum rate of rise (V_A) after the muscle had recovered in normal Ringer solution is about 400 V/s in this fibre which should be compared with the control value of about 600 V/s. This indicates that the effect of the detergent at this concentration (160 μM) is not fully reversible although the fibre was allowed to recover in normal Ringer solution for 75 min. This is also illustrated

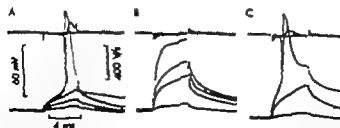


Fig. 2. Records from frog muscle fibres showing the change in action potential (upper trace) and rate of rise (V_A) produced by TX 100 (160 μM). To obtain uniform activation conditions, the fibres were hyperpolarized to -90 mV for about 30 s before eliciting an action potential. A. Normal Ringer solution. After 20 min in TX-100, 160 μM . B. After 75 min in normal Ringer solution.

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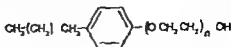
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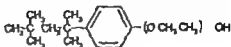
TN 101 is a polyoxyethylene nonylphenol (Fig. 1) with a n between 9 and 10 and a HLB number of 13.4 (Rohm & Haas Surfactants, Handbook of Physical Properties CS-Ola, Rohm & Haas Co, Philadelphia). The detergents were solved in the Ringer solution to the actual concentrations.

Results

TX-45, TX 100 and TN 101 produced no gross morphological changes as observed in the dissecting microscope and no spontaneous contractions could be observed for the con-



TRITON III



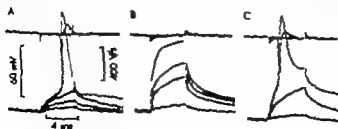
TRITON X

Chemical formulae for the detergents in the 4 Triton X series. Indicates the number of ethylene units.

used in the present study (*i.e.* 10–320 μM). The effects of TX 100 were primarily of TX-45 and TN-101 were later applied at a few concentrations and the effects by these detergents compared to those of TX-100. The resting membrane properties of using the resting membrane potential (E_r), the specific membrane resistance, the membrane capacitance (C_m). The maximum rate of rise (V_{max}) was used to analyze the action potential (AP). This parameter was chosen as it is a good approximation of sodium current (I_{Na}) underlying the muscle action potential.

Triton X 100

The effect of TX 100 on the muscle action potential is illustrated in Fig. 2 for three fibre traces at different times. Record (A) shows the AP in normal Ringer solution. After 160 μM TX 100 the muscle fibre AP is completely abolished (B) and the changes produced by the current pulses reflect the passive properties of the fibre. After 20 min the muscle was returned to normal Ringer solution and about an almost normal AP could be obtained (C). However the differentiated signal clearly shows that the recovery was not complete. The maximum rate of rise of the muscle had recovered in normal Ringer solution is about 400 V/s in this fibre could be compared with the control value of about 600 V/s. This indicates that the detergent at this concentration (160 μM) is not fully reversible although the as allowed to recover in normal Ringer solution for 75 min. This is also illustrated



Traces from frog muscle fibres showing the change in action potential (lower trace) and rate of rise (upper trace) produced by TX 100 (160 μM). Traces obtained under identical conditions, the fibres were held at -90 mV for about 30 s before eliciting an action potential. A. Normal Ringer solution. B. 160 μM TX 100. C. After 75 min in normal Ringer solution.

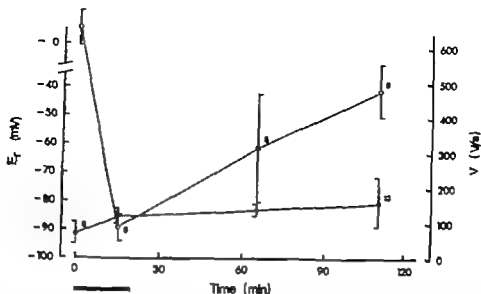
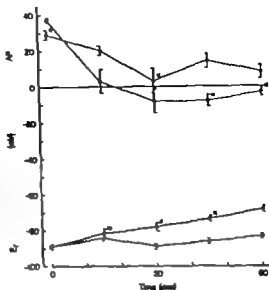


Fig. 3 Resting membrane potential (E_r) and maximum rate of rise (V_A) vs time for muscle exposed to $160 \mu\text{M}$ Triton X 100 for 20 min (bar) and allowed to recover in normal Ringer solution for 100 min. The mean E_r and V_A are plotted in closed and open circles respectively. The bars are the standard deviation (S.D.) of the mean. The number of fibres used to estimate mean and S.D. are indicated by figures at each point. The mean and S.D. for 15 min was calculated from measurements between 10 and 20 min, the mean and S.D. for 67 min was calculated from measurements between 64 and 70 min and the mean and S.D. for 112 min was calculated from measurements between 104 and 120 min.

In Fig. 3 which shows result from several fibres of a muscle which was treated at $160 \mu\text{M}$ for 20 min and allowed to recover in normal Ringer solution for 100 min. As can be seen the resting membrane potential (E_r) is only slightly affected during the exposure the fibres depolarised 6 mV (from -91.5 to -85.5 mV after 15 min) and during recovery there was an additional depolarization of about 5 mV the mean E_r at 112 min being -80.0 mV. In contrast, the effect on V_A was pronounced. From a control value of 655 V/s, V_A was reduced to 84 V/s after 15 min in TX 100 following recovery for 100 min in normal Ringer solution V_A was 480 V/s. The maximum value for a recovered fibre was obtained after 119 min, V_A being 560 V/s. It is thus uncertain if the effect is completely reversible or not. In any case the time required for complete recovery appears to be considerable.

To study in more detail the effect of TX 100 several concentrations from 10 to $320 \mu\text{M}$ were tested and the time course of the changes of E_r and the action potential (AP) were followed. In Fig. 4 the effect on E_r is shown before and during exposure at two concentrations (40 and $160 \mu\text{M}$). As seen the E_r value decreases linearly with time of exposure. In 60 min E_r decreased to -83 mV at $40 \mu\text{M}$ and to -68 mV at $160 \mu\text{M}$. Measurements (not illustrated in Fig. 4) at $80 \mu\text{M}$ and $320 \mu\text{M}$ showed that at the former concentration E_r was not reduced (from -88.0 to -88.0 mV in 60 min) and at the latter concentration there was a linear reduction in E_r from -89.0 mV to -68.6 mV. This suggests that increasing the concentration from $160 \mu\text{M}$ to $320 \mu\text{M}$ does not influence the rate of decrease in E_r . The reduction in E_r is thus not related to the concentration of TX 100 in a simple way.

The specific membrane resistance (R_m) and the membrane capacitance (C_m) were followed in one muscle exposed to $40 \mu\text{M}$ TX 100. A slight increase in R_m from $2577 \pm 622 \Omega\text{cm}^2$

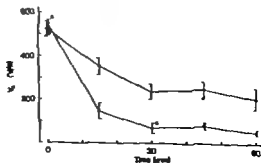


Resting membrane potential (E_r) (upper curves) and peak height of action potential (V_p) (lower curves) vs. time for muscle bathed with TX 100 for 60 min at doses of 40 μ M (filled circles, solid line) and 160 μ M (open circles, broken line). Muscles were exposed at 40 μ M and then at 160 μ M, each providing 20 to 40 surface fibers. Mean values \pm S.D. from the tissues were pooled and mean and standard deviation (S.D.) calculated. Number of cells are indicated by figures at each point.

to $3.790 \pm 1.162 \Omega \text{cm}^2$ ($n=5$) was obtained. Values are mean \pm standard error of the mean (S.E.). However statistically tested at a 5% level this difference is not significant. τ_m was estimated using the relation $C_m = R_m \tau_m$ where τ_m was taken as the time for an ionic potential change to reach 83% of full value (Hodgkin and Rushton 1946). C_m was found to be $13.3 \pm 4.5 \mu\text{F}/\text{cm}^2$ ($n=4$) in normal Ringer solution and $10.3 \pm 2.9 \mu\text{F}/\text{cm}^2$ after 60 min in TX 100 (mean \pm S.E.). This difference is not statistically significant at 5% level. The results suggest that TX-100 has little effect on passive permeability properties and that E_r linearly decreases with time at concentrations greater than 80 μ M.

The effect of TX 100 on the dynamic membrane properties is illustrated in Fig. 4 (height of action potential) and in Fig. 5 (V_p). In contrast with the almost linear time course of decrease of E_r , changes in height of the AP and V_p seem to follow a more exponential time course with a steady state reached after about 30 min. It is most likely that the detergent affects E_r and V_p through different mechanisms.

Similar experiments following V_p for other concentrations from 10 to 320 μ M were per-



Maximum rate of rise (V_p) vs. time for muscle bathed with Triton X 100 at 40 μ M (filled circles, solid line) and 160 μ M (open circles, broken line). Points are mean values and bars indicate \pm S.D. of mean. Figures above or below the points are number of fibers.

TABLE I: Equilibrium inhibition of V_r by Triton X 100.

V_r (mV)	V_0	$\frac{1-y_r}{y}$	[TX 100] (M) 10^{-6}	\log [TX 100]	$K_D (-1)$ (M) 10^{-6}	$K_D (-1.57)$ (M) 10^{-6}
76	0.76	9.00	10	-5.00	90	12.7
74	0.74	8.5	20	-4.70	57	11.9
68	0.55	0.82	40	-4.40	33	10.2
55	0.35	0.18	80	-4.10	14	6.7
51	0.27	0.15	160	-3.80	24	16.5
23	0.27	0.04	320	-3.50	12	13.0
Mean \pm S.D.					38 ± 30	11.8 ± 3.3

standing is equation (3). The mean \pm S.E. was $38 \pm 12 \mu\text{M}$, but it is noted that the individual K_D values vary appreciably the highest values being associated with the lowest concentration of the detergent, thus indicating that probably is not 1:1 evaluation. Hill plot (Hill 1909; Aronson, Jermolovs and Van Rossum 1964) was used (Fig. 7). In this graph $\log(1 - y_r/y_0)$ versus \log (TX 100) was plotted and calculated, its value being 1.57 (correlation coefficient = 0.99). This value deviates considerably from 1. Le the reaction is of the type involving one ligand to one receptor site. K_D for -1.57 was also computed and entered in Table I, the mean \pm S.E. being $11.8 \pm 1.3 \cdot 10^{-6} \text{ M}$ which is equivalent to $38 \mu\text{M}$ for 50 percent in V_r . These findings indicate that if there exist two sites in the classical sense they are more likely interacting. However other mechanisms are possible which will be discussed later.

Comparison between TX-45 TX 100 and TN 101

In connection with the experiments with TX-100 some other detergents of the Triton series were studied for comparison with respect to their effect on E_r and V_A . The time course of the effect on E_r of TX-45 TX 100 and TN-101 is shown in Fig. 8. The standard deviation (S.D.) is less than $\pm 8.0 \text{ mV}$ the mean value for the S.D. for all E_r being 4.8 mV . It appears from Fig. 8 that TX 100 has greater effect on E_r than TX-45. It is of interest to note that the slope of the regression line for TX-45 does not differ from zero, indicating that this detergent does not affect E_r .

In contrast to the effect on E_r all three detergents strongly reduce V_A (Fig. 9), this parameter being reduced to almost zero at 60 min for all detergents. It cannot be excluded that the effect differs somewhat with respect to the time course. The curves were drawn by eye and S.D. omitted as in Fig. 8. The mean absolute value of the S.D. for all points in this graph was 61 V/s with the maximum value for the S.D. being $\pm 115 \text{ V/s}$. It is obvious that the detergents differ in their action on the frog muscle membrane, this difference being most marked with respect to their effect on E_r .

Discussion

The present study has provided evidence that nonionic detergents in the Triton series abolish the action potential in the frog muscle with small or no effects on the resting membrane potential. Passive electrical properties such as the specific membrane resistance (R_m) and the membrane capacitance seem to be very little altered by these detergents. The static ionic properties of the TX 100-membrane system (depression of AP) were determined in

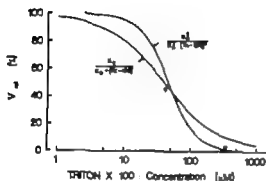


Fig. 6

Fig. 6. Relationship between maximum rate of rise and concentration of Triton X 100. $V_{A,rel}$ is the relation between V_A after exposure to the actual concentration for 30 min and V_A before the application. Points indicate measured values. Curves are $(1-y_p)$ (see text) for 2 values of n : $n=1$ (first order reaction) and $n=2$ (two TX 100 molecules reacting simultaneously with two sites). For additional information, see text.

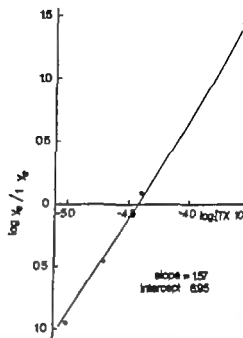


Fig. 7

Fig. 7 Hill plot. $\log y_p/(1-y_p) = \log (TX\ 100)$. Correlation coefficient $r=0.98$.

formed to obtain a dose-response relationship. In Fig. 6 is plotted the reduction in maximum rate of rise (V_A) after 30 min ($V_{A,rel}$) versus concentration of TX 100. From this graph the concentration for 50% reduction of V_A (ED_{50}) was found to be $36\ \mu M$ which is equivalent to the value of the apparent dissociation constant ($K_{D,app}$).

Steady state kinetics

To account for the effect of TX 100 on V_A it was assumed that TX 100 binds reversibly to some acceptor in the muscle membrane, probably a receptor. It was further assumed that one or more molecules of TX 100 simultaneously react with this site (cf. Hubbard, Linds and Quastel 1969) i.e.



where k_1 and k_2 are the association and dissociation rate constants respectively. It is assumed that V_A in the presence of TX 100 is proportional to the fraction of unoccupied sites, $1-y_p$, where y_p is the fraction of receptors occupied at equilibrium. From the law of mass action the occupied fraction of receptors at equilibrium is given by

$$y_p = \frac{[TX-100]^n}{[TX-100]^n + K_D} \quad (\text{cf. Hubbard et al. 1969; Rang 1971}) \quad (2)$$

From this the equilibrium dissociation constant $K_D = k_2/k_1$ can be calculated according to

$$K_D = (1-y_p)/y_p \cdot (TX\ 100)^n \quad (3)$$

Table I contains values for y_p and $(1-y_p)/y_p$, i.e. the ratio of unoccupied sites to occupied sites. From this ratio and the concentration of TX 100 the equilibrium dissociation constant K_D for $n=1$ was calculated.

associated to the membrane may also be the target for detergent interactions. It is well known that detergents bind to both water soluble proteins and to more hydrophobic proteins, both in a ligand-receptor fashion according to the law of mass action or cooperatively with many detergent molecules interacting simultaneously (Helenius and Sauer 1975, Tanford and Reynolds 1976). This association will often be followed by denaturation of the protein although this does not seem to be the case using "mild" detergents such as Triton X 100. Nevertheless, many membrane-bound proteins loose their biological activity without being denaturated which may either be due to a ligand-receptor action or a perturbation of the lipids in the vicinity of the protein by hydrophobic interaction between detergent and protein. The microenvironment of the proteins will thus change and will have consequences for their function (Tanford and Reynolds 1976).

As mentioned in the Results changes in E_r are not related to the concentration of Triton X 100 in a simple fashion. For concentrations up to 80 μ M where the maximum rate of rise (V_A) is reduced to about 15 % of control (Fig. 6) a very small reduction in E_r is observed, while at 160 μ M a linear depolarisation with time from -88.6 mV to -67.5 mV in 60 min is found. Together with the fact that R_m and C_m at 40 μ M TX 100 is approximately unchanged it is obvious that the passive permeability properties of the muscle membrane are little affected up to 80 μ M of TX 100. If it is assumed that the lipid bilayer is mainly responsible for these passive properties (cf. Singer and Nicolson 1972) it can be concluded that the association of TX 100 to the membrane, does not impart any drastic changes in the physical properties of the lipid core, the resting permeabilities for different ions probably being normal. However it is reported that Na-K-ATPase looses its biological activity after treatment with TX 100 (Tanaka and Abood 1964). A reduction of the pumping activity in the muscle membrane at high concentrations could well account for the observed slow linear depolarization seen in Fig. 8 (Draper, Friebe and Karmel 1963, Locke and Solomon 1967). The effect of TX 100 on the action potential is most likely to be attributed to a reduction of the initial sodium current as suggested by the measurements of V_A . The apparent dissociation constant K_{app} found in Fig. 6 is 36 μ M and the corresponding value for K_D calculated from eq. (3) with $\alpha = 1.6$ is 38 μ M. The concentrations are in the same range as local anaesthetics, e.g. lidocaine reduces the sodium current in myelinated nerve fibres to 50 % at 100 μ M (Hädic 1966) and it is demonstrated that procaine reduces the V of chicken heart muscle fibres to 50 % at a concentration of about 100 μ M (Josephson *et al.* 1976). The binding characteristics of the detergent as revealed from the dose response curve of V_A make it unlikely that the binding reflects an unspecific (i.e. hydrophobic) interaction with the membrane lipids, since this would imply effects on the passive permeability which are demonstrated to be very small. A more plausible explanation is that the dose response curve reflects some interaction with proteins in the membrane eventually involved in the generation of the action potential. Such interaction is probably not of the cooperative type, referred to above, since it would give a much steeper dose response curve (Tanford and Reynolds 1976). The interaction can be better explained if it is assumed that the protein has some sites for the detergent, which may not necessarily be stereospecific but perhaps of the "group-specific" type (Monod, Wyman and Changaux 1965) with lower affinity. In view of the fact that many proteins integrated in the membrane have hydrophobic regions which may establish nonpolar inter-

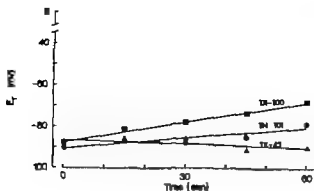


Fig. 8 Resting membrane potential as time after application of three detergents at a concentration of 10^{-6} M. Bars indicating \pm S.D. are omitted for (see text). Straight lines are fitted points by a least square regression. The measurements were made from 2 (TX-45 and TX-100) and 3 muscles (TN-101).

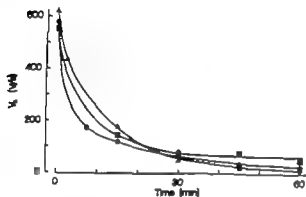


Fig. 9 Maximum rate of rise (V_A) after application of TX-45 (triangles), TN-101 (squares) and TX-100 (circles) at a concentration of 10^{-6} M. Bars indicating S.D. been omitted for clarity (see text). Curve drawn by eye. Same number of muscles as in Fig. 8.

two ways. It was not possible to describe the process as either a first or second order reaction and n was estimated to 1.6.

The action potential in the frog skeletal muscle fibre is the result of a fast inward sodium current followed by a slowly developing outward potassium current (Adrian, Chandler and Hodgkin 1970). Under physiological conditions V_A is a good estimate of the inward current (Hodgkin and Katz 1949, Schwartz, Ulbricht and Wagner 1973). Theoretically changes of V_A observed in the present study may be equivalent to one of following changes of ionic currents: i) a decrease in sodium current, ii) an increase in potassium current, iii) summation of both. The small alterations in potassium current on the frog nerve caused by TX-100 (Brisson and Rydqvist, to be published) suggest that changes in V_A represent mainly the changes in sodium current.

As a background for discussing the present findings a brief summary of possible interactions between amphiphilic molecules and biological membranes will be given. Amphiphiles such as detergents are known to interact both with lipids and proteins although in different ways. The interaction between detergents and phospholipids, which is the main lipid constituent of biological membranes, may result in lyotropic mesomorphism and different liquid crystalline phases with diverse physical properties may occur mainly depending on the ratio of the components involved (Small 1968, Ekwall 1975). The liquid crystalline phase is the lamellar phase known to be present in the lipid bilayer in biological membranes (Small, Bourges and Dervichian 1966, Small 1968). The detergents may alter the naturally occurring lipid state, e.g. from a fluid liquid crystalline state to a less fluid one or even to a solid gel phase thus changing physical and consequently physiological properties of the lipid bilayer.

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actions with the detergent it is conceivable that the detergent may substitute for naturally occurring membrane lipids. This would require more "sites" for TX 100 than is predicted from the steady state kinetics. In terms of classical occupancy theory the observed is compatible with the assumption of two sites which are mutually interacting. An alternative possibility that could account for the shape of the dose-response curve is the existence of an intermediate step between the primary interaction and the actual effect. Rang (1977) proposed that the shape of the dose-response curves could be accounted for if several groups of m sites must be occupied to exert the effect. A more likely explanation is that the effect seen, is a result of the interaction between the detergent and two or more distinct sites which mutually influence their affinity for the detergent, i.e. allosteric theory (Nelson *et al* 1965). However the present findings cannot distinguish between these alternatives.

Finally a comparison between the effects of the three detergents used (TX-45 T and TN 101) reveals little difference in their action on the frog muscle membrane. The clear difference is that TX-45 which is a more hydrophobic detergent (HLB number does not seem to depolarize the cell membrane. This may be accounted for by a different interaction with the membrane lipid phase due to its hydrophobicity. An equally satisfactory explanation may be that this detergent does not interfere with the sodium-(Na-K-pump).

In summary the detergents TX 100, TX-45 and TN 101 seem capable of abolishing action potential in muscle by reducing the early sodium current without any drastic change in passive electrical properties. It is suggested that this effect is mediated via a perturbation of one or more proteins in the membrane either in a drug-receptor mechanism involving two (or more) sites or a more unspecific interaction to hydrophobic loci on the protein.

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Methods

was operated under ketamine-prochlorperazine anaesthesia, with strict aseptic precautions. The right carotid artery and the right external jugular vein were cannulated with silicone rubber catheters, their tips being in the aortic arch and the right atrium, respectively. The other catheter leads were drawn out through the skin over the back of the skull. The latter was opened over the occipital and parietal lobes. The superior sagittal sinus was removed, except for the frontal lobes and the diencephalon after which the brain was cut between the colliculi with blunt spatula.

On subsequent days the animals received electrolytes, glucose solution, amino acids and lipid suspension (Pleasanton), serum potassium and sodium were followed daily and, if necessary corrected. Oxygen was given by face mask, regulated by rectal thermometer keeping body temperature at about 35°C. Apnoeal observations were, however performed at body temperature of 38°C.

For 1-7 days, the animals were tracheostomized and cannula with thermocouple was put in the trachea permitting recording of the ventilation rate. Arterial pressure was monitored by connecting the catheter to a P23 AC transducer and heart rate was obtained by an ordinate writer triggered by the catheter. All recordings were made on Grass polygraph. In 2 cats, the vagi were dissected free in the neck so that they could be cut in the course of the experiment.

When a head submersion drive was to be performed, the animal was put with the ventral side down on a board, its head over the edge. A jar with water was lifted up from below until the nose and eyes were submerged while the animal could ventilate freely through the tracheal cannula.

In some cats, 1 ml of ether (15-20°C) was instead injected into nostril through this polyethylene tube. The animal was then lying on its side and was not tracheostomized.

In addition, 3 cats were acutely decerebrated under ether anaesthesia, to be used in head submersion experiments on the same day.

In all experiments the brain stems were fixed in formalin and paraffin-embedded. Sections, 20 μ m thick, were stained with the Luxol fast blue-masson red method. The sections were inspected under microscope to confirm that the sections had been completely cut at the intended level, between the colliculi.

Results

Chronically decerebrated cats were subjected to head submersion. Of these 9 responded with apnoea and bradycardia whereas 2 cats did not display this type of responses. The latter cats were tested already on postoperative day 1 and 2, as compared to a mean of 5.4 ± 1.7 days for the others. In 7 animals, the reflex response was entirely reproducible throughout the experiment. In the other 2 (postoperative day 7 and 3), it was observed in about half of the tests.

Arterial blood pressure was maintained or slightly increased during the "dives". Apnoea and bradycardia always occurred together (see Fig. 1). The latency of the response was 5 s which could vary even in the same animal but was usually below 15 s. Thus, the touching of the face did not immediately elicit the reflex response. Instead, it seemed often to occur first after some water had reached the nasal cavity or possibly other parts of the respiratory passages. Once the response had started, however it was sustained and lasted during a submersion of up to 60 s. After interruption of the "drive" apnoea and bradycardia vanished within 10 s. The reflex adjustments were not elicited by postural changes as the animals kept the same position throughout the tests. The responses occurred independently of water temperature (10-30°C).

In contrast, 3 acutely decerebrated cats subjected to submersion of the head displayed neither apnoea nor bradycardia.

The reflex bradycardia was very prominent in the chronically decerebrated cats. In

Apnoea and Bradycardia from Submersion in "Chronically" Decerebrated Cats

By

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Abstract

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In chronically but not acutely decerebrated cats, submersion of the head caused apnoea and war bradycardia, associated with a maintained or slightly raised arterial pressure. Since these reflex adjustments though very reproducible, occurred with varying latency and could be induced also by nasal injection water they appeared to be, at least in part, elicited from the upper respiratory passages. Thus, a terrestrial mammal, reputed to abundantly form of submersion, can exhibit adjustments during head submersion, similar to those habitually diving species. This response pattern is basically organized at the lower brainstem level.

Submersion in water causes apnoea, bradycardia and peripheral vasoconstriction, not only in air-breathing aquatic animals but also in terrestrial species such as dog and man. In the duck this response pattern can be elicited by stimulation of receptors in the upper respiratory passages (Blix, Rettedal and Stokkan 1976). The resulting adjustments appear to be the result of a complex interaction between the respiratory and cardiovascular centres (Angell-James and de Burgh Daly 1972 a, Blix 1975).

In the duck the basic elements of the diving response remain after decerebration (Huxl 1913, Andersen 1963, Djojosingito, Folkow and Yonce 1969). However, it has been inferred that cortical influences are prominent in mammals and may even dominate the event. Thus, seals often display an anticipatory bradycardia before a dive and also increase the heart rate when approaching the surface (Jones *et al.* 1973). Further, if the animal decides to interrupt the ascent and continue the dive, the bradycardia may return (Murdaug, Seabury and Mitchell 1961).

Therefore, it appeared of interest to study whether the isolated brain stem of mammals is capable of mediating a well organized diving reflex and to what extent this is the case also in notorious non-divers. For such purposes "chronically" decerebrated cats were exposed to simulated diving by means of head submersion.

proposed that the corresponding reflex adjustments in ducks are elicited by receptors in the outside of the head. However, more recent studies suggest that receptors in the respiratory passages, especially in the glottis region, are of particular importance (Barnford *et al.* 1974, Blix, Rettedal and Stokkan 1976). In the duck such receptors are immediately sealed by the beak as it cannot completely close the beak (Blix, Rettedal and Stokkan 1976). In conscious trained dogs, voluntary snout immersion causes apnoea and bradycardia (Jones, Stone and Young 1974). Water flow through the nose gives the same effect in anaesthetized dogs (Angell James and de Burgh Daly 1977a, b). It is likely that submersion in water activated similar receptors in the decerebrated cats. To elucidate this, water was forced into one nostril which caused immediate apnoea and bradycardia in some cats. In the context it should be noted that several other stimuli, besides water when applied to the nasal cavity can elicit a similar response pattern. Thus cigarette smoke causes apnoea and bradycardia in the rabbit and this is still the case after decerebration (White and McRitchie 1973).

The apnoea and bradycardia always occurred together in the present experiments. The decrease in heart rate took place immediately after the onset of apnoea even if it tended to become modified during prolonged submersion. Several links between the apnoea and bradycardia have been envisaged (*cf.* Angell James and de Burgh Daly 1977a). Thus, the water sensitive receptor may elicit a primary reflex excitation of the vagal cardioinhibitory fibres. But the bradycardia can also be induced *secondarily* either by direct pathways between the respiratory and cardiovascular neuron pools in the brain stem or via reflexes from pulmonary and bronchial receptors. The bradycardia may be enhanced by an increased activity in unmyelinated vagal afferents from cardiac receptors, activated by the peripheral vasoconstriction (Blix, Wessergren and Folkow 1976). During prolonged submersion, arterial chemoreceptors are brought into play by the gradual fall in blood oxygen content, still more modifying the bradycardia as long as apnoea prevails. As several of these reflexes have been afferent pathways in the vagi, vagotomy would have the double effect of abolishing both the afferent and several afferent links in this integrated reflex response. It is therefore not surprising that vagotomy greatly changed the reflex response to head submersion, with a reversal of the bradycardia to a weak tachycardia.

Thus, submersion of the head of decerebrated cats caused apnoea and bradycardia, indicating that these reflex effects do not require higher levels of the nervous system. This certainly does not deny that this basic submersion response can be considerably modified by higher nervous levels in intact animals. Indeed, there is ample evidence that this is the case, especially in diving species (Mordhaugh, Seabury and Mitchell 1961, Jones *et al.* 1973).

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Thanks are due to Mrs Kerstin Bengtsson for skilful technical assistance.

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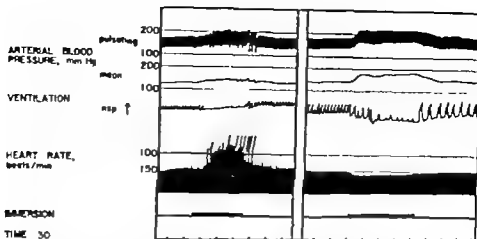


Fig. 1 Cat, 2.2 kg, postoperative day 3. In the first panel, submersion of the head causes apnoea and bradycardia with some latency. After cutting of the vagi in the neck (second panel), the submersion apnoea is interrupted by inspiratory gasps and the bradycardia is abolished.

the majority of expts. heart rate fell from 150–200 beats/min to below 100. During the prolonged submersions, there was an accentuation of the bradycardia after 15–20 s.

Bilateral vagotomy abolished the bradycardia during the apnoea and there was instead moderate tachycardia and an intensified rise in arterial pressure (2 cats). In the experiment illustrated in Fig. 1 the apnoea was entirely reproducible and sustained during 8 trials before vagotomy. After cutting of the vagi, however, there were inspiratory gasps during submersion.

4 cats were exposed to water instillation in the nasal cavity. In 2 cats (postoperative day 1 and 2), this immediately elicited apnoea and bradycardia lasting for 10–25 s, whereas in the others, no responses were elicited (postoperative day 4). The 2 responsive animals were some days later tracheostomized and subjected to head submersion which regularly elicited apnoea and bradycardia.

Discussion

In the majority of the "chronically" decerebrated cats, submersion of the head caused respiratory arrest and marked bradycardia. Similar findings have previously been made in acutely decerebrated ducks (Huxley 1913; Andersen 1963; Djojosingito, Folkow and Yonce 1969). These adjustments, very much reminding of those occurring during spontaneous diving in several species, can thus be mediated by lower levels of the central nervous system, not only in the duck but also in a mammal that is certainly far from being a habitual diver. These responses were, however, not observed in acutely decerebrated cats, but only in animals allowed to recover for some days after the truncation. Apparently decerebration entails a reversible trauma to the central nervous system, besides the obvious irreversible injuries (Monakow 1914, p. 26).

It is not clear exactly which type of receptors that were responsible for the reflex response to submersion in the present expts. It did not always occur immediately after submersion but appeared instead to be related to water reaching the upper respiratory passages. Andersen

proposed that the corresponding reflex adjustments in ducks are elicited by receptors outside of the head. However more recent studies suggest that receptors in the respiratory passages, especially in the glottis region, are of particular importance (Barnford *et al.* 1974, Bliz, Rettedal and Stokkan 1976). In the duck such receptors are immediately stimulated by water as it cannot completely close the beak (Bliz, Rettedal and Stokkan 1976). In conscious trained dogs, voluntary snout immersion causes apnoea and bradycardia (e.g. Stone and Young 1974). Water flow through the nose gives the same effect in trained dogs (Angel James and de Burgh Daly 1972 b). It is likely that submersion activates similar receptors in the decerebrated cats. To elucidate this, water was forced into one nostril which caused immediate apnoea and bradycardia in some experiments. It should be noted that several other stimuli, besides water when applied to the nostrils can elicit a similar response pattern. Thus cigarette smoke causes apnoea and bradycardia in the rabbit and this is still the case after decerebration (White and McRae 1973).

Apnoea and bradycardia always occurred together in the present experiments. The decrease in rate took place immediately after the onset of apnoea even if it tended to become fixed during prolonged submersion. Several links between the apnoea and bradycardia have been envisaged (*cf.* Angel James and de Burgh Daly 1972 a). Thus, the water sensitive receptors may elicit a primary reflex excitation of the vagal cardioinhibitory fibres. But the bradycardia can also be induced *secondarily* either by direct pathways between the respiratory and cardiovascular neuron pools in the brain stem or via reflexes from pulmonary and chemoreceptors. The bradycardia may be enhanced by an increased activity in unmyelinated vagal afferents from cardiac receptors, activated by the peripheral vasoconstriction (Wessergren and Folkow 1976). During prolonged submersion, arterial chemoreceptors are brought into play by the gradual fall in blood oxygen content, still more enhancing the bradycardia as long as apnoea prevails. As several of these reflexes have different pathways in the vagi, vagotomy would have the double effect of abolishing one afferent and several afferent links in this integrated reflex response. It is therefore surprising that vagotomy greatly changed the reflex response to head submersion, with replacement of the bradycardia to a weak tachycardia.

Thus, submersion of the head of decerebrated cats caused apnoea and bradycardia, indicating that these reflex effects do not require higher levels of the nervous system. This certainly does not deny that this basic submersion response can be considerably modified by higher nervous levels in intact animals. Indeed, there is ample evidence that this is the case, especially in diving species (Mordasough, Seabury and Mitchell 1961, Jones *et al.* 1973).

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Role of the Adrenergic Nervous System in Development of Training-Induced Bradycardia

By

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Abstract

SÖVÄRDEN, K., E. SVANFELDT and Å. KILBOM. Role of the adrenergic nervous system in development of training-induced bradycardia. *Acta physiol. scand.* 1977 101 481-488

Sprague-Dawley rats, normal and chemically sympathectomized with 6-hydroxy-dopamine, were trained by tread-mill running. The normal rats, unlike the sympathectomized animals, showed reduction of the exercise heart rate after the training period. Compared to a sedentary control group the sympathectomized rats showed no difference in intrinsic heart rate after pooling and denervation and no increase in heart weight. The increase of the heart weight/body weight ratio after training was smaller in the sympathectomized group than in the normal ones. The results show that functioning adrenergic nervous system is necessary for its plastic adaptation to physical training. Administration of noradrenaline to pooled trained and untrained rats showed that beta-adrenergic receptor sensitivity was not altered by physical training. The intrinsic heart rate of normal trained rats was lower than that of normal control rats.

Key words: Physical training, intrinsic heart rate, 6-hydroxy-dopamine, receptor sensitivity, pooling.

The physiological adaptation to physical training includes a variety of adjustments in the mechanisms of uptake, transport and utilization of oxygen.

The main changes in cardiac performance after training are decreased heart rate and an increased stroke volume at rest and during exercise (cf Åstrand and Rodahl 1970). However the mechanisms producing these adjustments are still poorly understood. It has been shown that the activity of the autonomic nervous system is altered during training and several authors claim that these changes are involved in the development of training bradycardia.

Thus the training bradycardia has been attributed to an increased cardiac concentration of somatostatin (Tipton and Taylor 1965) or to a decreased cardiac catecholamine concentration (de Schryver *et al.* 1967, 1969). Östman and Sjöstrand (1971) however using a far more intensive training program, found no difference in cardiac noradrenaline concentration between trained and untrained rats.

In training experiments where autonomic blockade was performed the sympathetic activity seemed to have decreased after training (Ekblom *et al.* 1973b, Frick *et al.* 1967, Herrlich *et al.* 1960).

The importance of the autonomic nervous system for the development of training bradycardia can also be analyzed by eliminating the different constituents of the autonomic nervous system in animals. If these animals are trained, and compared to a group of trained, nontreated controls, the character of any differences in the adaptation to physical training can be used to estimate the significance of the excluded parameter.

In this study the principal aim was to elucidate the role of the adrenergic system in the adaptation to physical training, mainly the post training bradycardia. The method chosen was to study the heart rate response to training both in normal rats and in rats subjected to sympathectomy. The sympathectomy was induced chemically with 6-hydroxy-dopamine (6-OH DA), which causes a long lasting degeneration of the adrenergic nerves (de Champlain and Nadeau 1971, Malmfors and Thoenen 1971, de Champlain and van Ameringen 1972, Gauthier *et al.* 1972).

The heart rate effect of physical training may not be entirely attributable to changes in autonomic nerve activity. The "intrinsic heart rate"—whether it can be explained by hypertrophy, increased contractility or in other terms—may also be influenced by physical training. In this study pithing (Shipley and Tilden 1947) was performed in order to eliminate the cerebral and spinal influence on the circulatory system. It was used in combination with 6-OH DA treatment and bilateral vagotomy in both the normal and the previously treated rats in order to obtain a preparation completely depleted of nervous influence on heart rate, the heart still remaining in the circulatory system of the body.

Some authors have suggested that the sensitivity of the adrenergic beta-receptors decreases during physical training. Crews and Aldinger (1967) when measuring isometric systolic tension in anesthetized rats, found that hearts of trained rats responded less to a given dose of exogenous noradrenaline than did those of untrained rats. Our finding (Ekblom *et al.* 1973a) of a less pronounced acute effect of 6-OH DA in trained rats also seemed to support this theory. Pavlik *et al.* (1976) however have found experimental support for a lower beta-activity but no marked changes in receptor sensitivity after physical training. In the present investigation, noradrenaline in increasing concentration was administered to rats previously subjected to pithing, 6-OH DA treatment and vagotomy in order to determine the heart rate reaction to noradrenaline when no nervous mechanisms could interfere.

Methods

25 female rats, of which 12 had been treated with 6-OH DA (50 mg/kg i.p.) as newborn (Nadeau *et al.* 1971) were trained by treadmill running at a speed of 11 m/min for 1 h a day 5 days a week, for 12 weeks. Training started when the animals were about 3 months old. Another 25 female rats, from the same litter as the exercised animals, served as controls. In this group, too, 12 of the rats had been sympathectomized a few days after birth.

Two months after birth, three small silver electrodes were implanted subcutaneously on the back of each rat. These electrodes could easily be connected to cables for ECG-recordings during exercise (Ekblom *et al.* 1973a).

Before and after the training period all rats were exercise tested and during three days prior to the test all rats were exercised for about 1 h a day in order to accustom them to treadmill running. Exercise heart rates were measured with the rats running on the treadmill at three different speeds, 12, 1 and 2.5 m/min respectively at an inclination of about 3°.

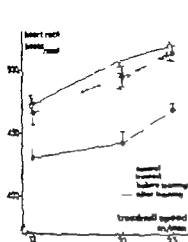


Fig. 1

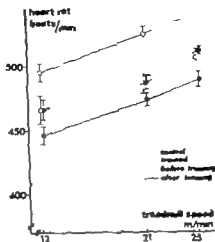


Fig. 2

Fig. 1 Normal rats. Heart rates during exercise at 3 different work loads before and after the training period. Mean values and S.E. are given. Open circles: control rats, filled circles: trained rats.

Fig. 2 Sympathectomized rats. Heart rates during exercise at 3 different work loads before and after the training period. Mean values and S.E. are given. Open circles: control rats, filled circles: trained rats.

The rats were exercised for 10 min at the lowest speed and subsequently for 5 min each at 21 m/min and 25 m/min. Heart rate was recorded in 10 periods (3 min) during the last 3 min of each work load, and the mean was used. During the training and testing period, 6-OH-DA treatment, 30 mg/kg was repeated every second week (de Groot 1971). After the final work tests, all rats, both normal and sympathectomized, were treated with 6-OH-DA, 30 mg/kg. On the following day they were anesthetized with ether and subjected to perfusion and bilateral cuts of the vagus nerves in the cervical region. The animals were artificially respiration and the external jugular vein was cannulated. A polyethylene catheter was also inserted into the aortic artery and connected to a Statham P 23 DC transducer for blood pressure recording. A Grass Polygraph. Through the venous catheter increasing doses of noradrenaline (NA, 100 μ g) were injected and the heart rate and blood pressure responses to these injections were recorded. The NA infusion lasted about 30 min, after which the hearts and adrenals were removed and weighed.

Statistical analysis

Wherever possible, t in comparisons of exercise heart rates before and after training, the t -test for paired observations was used. In other cases, t in comparisons of heart weights between groups, the significance between means for different groups was calculated using Student's t -test for independent observations. Level of significance, unless otherwise stated, $p < 0.05$.

Results

In the normal rats, the exercise heart rates of the training and the sedentary groups were similar before the training period. After training, the heart rates of the trained rats were significantly lower at all work loads than those of the controls and also compared to the training values (Fig. 1).

In the sympathectomized group the trained rats had lower exercise heart rates than the controls after the training period, the difference being 40–50 beats/min (same as in the normal

TABLE I

Group	Body weight, g	Heart weight	Heart weight/body weight, %	Adrenal weight, g	Adrenal weight/body weight
Normal control (n=12)	285±5	0.935±0.014	3.293±0.069	0.031±0.001	0.110±0.003
Normal trained (n=12)	283±4	1.079±0.026	3.811±0.062	0.034±0.001	0.119±0.002
6-OH DA control (n=12)	286±5	0.921±0.024	3.212±0.045	0.032±0.001	0.111±0.002
6-OH DA trained (n=10)	266±6	0.942±0.010	3.556±0.059	0.035±0.001	0.131±0.005

Body weight, heart weight and adrenal weight after the training period. Mean values and S.E. are given. The symbols indicate the significance of differences between the trained groups and their respective control group.

p < 0.05

p < 0.01

p < 0.001

group). However, the difference in this case was due not so much to a lowered heart rate in the trained rats as to a rise in the controls (Fig. 2).

At the beginning of the training period no differences in body weight existed between the groups (mean 242 g). During the training period all groups increased their body weight, but the trained 6-OH DA treated rats to a smaller extent than the other groups (Table I). On the other hand, the normal trained rats had a significantly greater heart weight than their controls. The heart weight/body weight ratio was significantly higher in both normal and sympathectomized trained rats compared to their respective control groups. The difference was however small between the sympathectomized groups and reflected the difference in body weight. The difference in the heart weight/body weight ratio between trained normal rats and trained sympathectomized rats was significant ($p < 0.005$), see Table I.

The weight of the adrenal glands of the trained, denervated group was significantly greater than that of the denervated control group ($p < 0.01$), while there was no significant difference between the normal groups.

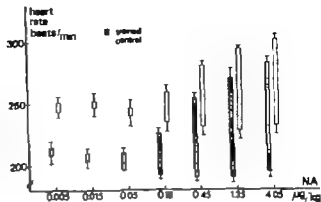
Concerning the adrenal weight/body weight ratio the difference between denervated trained and untrained animals was even more pronounced ($p < 0.001$) and there was a slight difference between the normal groups too ($p < 0.05$).

In the normal group the preinjection heart rates were significantly lower in the trained rats (Fig. 3). The magnitude of the HR increase in response to noradrenaline injection was the same in the trained and in the sedentary group. At some doses the HR increase was even slightly higher in the trained group (Fig. 3).

In the rats sympathectomized as newborn, the preinjection heart rates did not differ significantly between the trained and untrained groups. The HR response to exogenous noradrenaline was somewhat lower than that of the non-treated rats.

The blood pressure levels after phing were not significantly different in normal and sympathectomized rats. The elevation of systolic and diastolic blood pressure after nor

Fig. 3 Effect of NA infusion on heart rate of normal rats. The bottom of each column represents the pre-infusion heart rate \pm S.E. and the top represents the maximal heart rate response \pm S.E. The animals are pretreated with 6-OH-DA, vagotomized and poked before these measurements.



stimulus administration was the same in all four groups: previously sympathectomized and normal, trained and untrained (Fig. 4).

Discussion

In previous training expts. with rats, only heart rates at rest have been measured (Tipton and Taylor 1965; Pavlak *et al.* 1976). Other investigators have performed graded treadmill tests in rats, but not in connection with physical training (Barnard *et al.* 1974; Denimal *et al.* 1970; Vance *et al.* 1973).

When discussing the exercise heart rates before and after training in the different groups, the changes in body weight during the training period should be considered. Due to the increase in body weight of all groups during the training period, the post-training test as a 3° inclination must have been somewhat more strenuous. The effect seems however to have been modest, since the rats in the normal control-group had increased their exercise heart rate only slightly (5–10 beats/min, $p < 0.05$) after the training period. Likewise, it may be argued

Fig. 4 Effect of NA infusion on the diastolic blood pressure of normal rats. The bottom of each column represents the pre-infusion BP \pm S.E. and the top of each column the maximal pressure response \pm S.E. The animals were pretreated with 6-OH-DA, vagotomized and poked before these measurements.

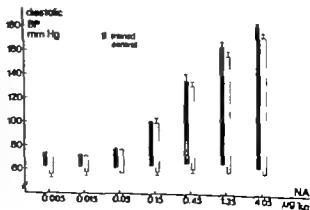


TABLE I

Group	Body weight, g	Heart weight, g
Normal control (n=12)	285±3	0.935±0.01
Normal trained (n=12)	283±4	1.079±0.02
6-OH DA control (n=12)	86±5	0.921±0.01
6-OH DA trained (n=10)	66±6	0.942±0.01

Body weight, heart weight and adrenal weight (g).
The symbols * indicate the significance of difference from control group.

p < 0.05

p < 0.01

p < 0.001

group). However the difference in this case was due to the trained rats as to a rise in the controls (Fig. 2).

At the beginning of the training period no difference was found between the normal and the trained groups (mean 242 g). During the training period the trained 6-OH DA treated rats to a smaller extent than the normal trained rats had a significantly greater heart weight/body weight ratio. This difference in heart weight/body weight ratio was significantly higher in the trained rats compared to their respective control groups. The difference in the heart weight/body weight ratio between the sympathectomized groups and the normal groups was significant (p < 0.005) (see Table I).

The weight of the adrenal glands of the trained, denervated rats was significantly higher than that of the denervated control group (p < 0.01) while no difference was found between the normal groups.

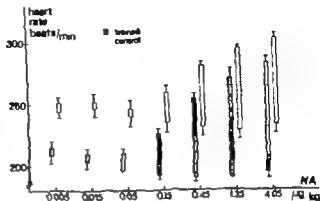
Concerning the adrenal weight/body weight ratio the difference between the trained and untrained animals was even more pronounced (p < 0.001) and the difference between the normal groups too (p < 0.05).

In the normal group the preinjection heart rates were similar in the normal and the trained rats (Fig. 3). The magnitude of the HR increase in response to the same dose of adrenaline was the same in the trained and in the sedentary group. At some doses the increase was slightly higher in the trained group (Fig. 3).

In the rats sympathectomized as newborn, the preinjection heart rates were significantly higher in the trained and untrained groups. The HR increase in response to adrenaline was somewhat lower than that of the non-treated rats (Fig. 3).

The blood pressure levels after pithing were not significantly different between the normal and the sympathectomized rats. The elevation of systolic and diastolic blood pressure was similar in the normal and the sympathectomized rats.

Fig. 3 Effect of NA infusion on heart rate of normal rats. The bottom of each column represents the pre-injection heart rate \pm S.E. and the top represents the post-injection heart rate \pm S.E. The animals were pretreated with 6-OH DA, vagotomized and paced before these measurements.



adrenaline administration was the same in all four groups previously sympathectomized and trained, trained and untrained (Fig. 4).

Discussion

In previous training expts. with rats, only heart rates at rest have been measured (Tipton and Taylor 1963, Pavlik *et al.* 1976). Other investigators have performed graded treadmill tests in man, but not in connection with physical training (Bernard *et al.* 1974, Denimal *et al.* 1970, Wipac *et al.* 1973).

When discussing the exercise heart rates before and after training in the different groups, the changes in body weight during the training period should be considered. Due to the increase in body weight of all groups during the training period, the post-training test at a 3 kcal/min must have been somewhat more strenuous. The effect seems however to have been modest, since the rats in the normal control-group had increased their exercise heart rate only slightly (5–10 beats/min, $p < 0.05$) after the training period. Likewise, it may be argued

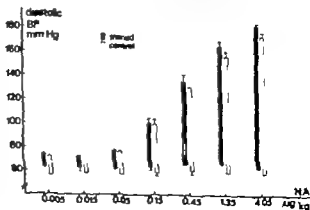


Fig. 4 Effect of NA infusion on the diastolic blood pressure of normal rats. The bottom of each column represents the pre-injection BP \pm S.E. and the top of each column the post-injection pressure \pm S.E. The animals were pretreated with 6-OH DA, vagotomized and paced before these measurements.

that since the exercise heart rates of the trained denervated rats were obtained at a body weight 10 percent lower than in the other three groups, the training effect in this group was somewhat exaggerated. It was actually only in the normal group that the exercise heart rate was significantly reduced after training. The lack of a similar effect in the *sympathectomized* group must be related to the 6-OH DA treatment. The sympathectomy did not impair the ability to increase heart rate, so the lack of training effect in these rats was not associated with an inability to reach high heart rates during training. On the contrary treated rats have been shown to have higher heart rates during exercise than normal rats (Ekblom *et al.* 1973a). Also at rest we have found a higher heart rate in 6-OH DA treated rats than in normal ones (unpublished results). The higher heart rate of denervated rats at rest and during exercise is not fully understood but some possible factors could be mentioned.

1. 6-OH DA treatment causes an adrenergic denervation of the blood vessels, resulting in an impairment of the circulatory control and a blood pressure reduction (de Champlain and van Ameringen 1972, Gauthier *et al.* 1972), which is partly compensated for by an increased adrenal catecholamine production.

A contribution to the high heart rate could come from the "supersensitivity" to catecholamines that has been shown to occur after sympathectomy (Nadeau *et al.* 1971).

2. Because of the impaired circulatory control the sympathectomized rats would require a greater cardiac output than normal rats to ensure the working muscles an adequate blood supply.

Thus, the increased heart rate of the denervated control group after the training period could reflect a progressive impairment of the circulatory function. In the denervated training group this impairment seems to have been counteracted by the training, although the adaptation to training was less efficient than in the normal trained group.

In the pitthed and denervated specimens there was no neural influence on heart rate and we have therefore used the term *intrinsic* for the heart rates obtained from these. In the present investigation the *intrinsic* heart rate was significantly reduced in the trained normal group whereas no difference in *intrinsic* heart rate was observed between trained and untrained sympathectomized rats.

Differences were also obtained in the development of heart weight and body weight between the trained sympathectomized and the trained normal group. Only the normal rats showed an absolute increase in heart weight after training compared to the respective sedentary group. The sympathectomized trained rats had a lower mean body weight than the other groups. Therefore, their heart weight-body weight ratio was greater than in the denervated control group although smaller than in the normal trained group.

Similar results have been reported by Tipton (1965) from measurements of heart weight, body weight and heart weight/body weight ratio in training expts. with immunosympathectomized rats.

All these parameters—heart weight and heart weight/body weight ratio, exercise heart rate and *intrinsic* heart rate—show that the abolition of the adrenergic nervous system by 6-OH DA treatment at birth and during the training period alters the adaptation of the circulatory system to physical training.

One or several factors affecting heart rate after pithing and denervation must have caused

difference between trained and untrained normal rats. Conceivable factors are intrasubject mechanisms, circulating adrenal catecholamines or other blood-borne agents, or variations of the receptor sensitivity to catecholamines. The hypothesis of a decreased isometric receptor sensitivity as a cause of the post-training bradycardia can be rejected, as the response to exogenous noradrenaline (increased heart rate and increased blood pressure in pithed rats) was the same in trained and untrained rats. The threshold dose, i.e. the lowest dose of noradrenaline which gives a significant response, was also the same in trained and untrained rats.

The alterations of the autonomic nerve function reported from investigations with autonomic blockade in trained animals and humans (see Introduction) do not seem to be the most fundamental explanation for the post-training bradycardia. This investigation has shown that trained rats still had their bradycardia after pithing, vagotomy and sympathectomy. This reduced intrinsic heart rate will have to be considered in the interpretation of results with autonomic blockade and it might explain the divergent opinions expressed by some authors, investigating the influence of training on autonomic nerve activity. Furthermore, the development of this reduced "intrinsic" heart rate through training seems to represent intact adrenergic nervous system, since in sympathetomized trained rats there was no such reduction.

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Vascular Reactivity to 5-Hydroxytryptamine (5-HT) in Hindquarter Vascular Beds, Aortic Strips and Portal Veins from Spontaneously Hypertensive and Normotensive Rats

By

LARS ÅHLUND, YEN LUNDQVIST, BOBEN SÖDERG and LILLIAN WEISS

Recent studies on spontaneously hypertensive rats (SHR) illustrate the hemodynamic importance of an altered precapillary vessel design in hypertension, extending earlier findings on renal hypertension in man. Thus, dose-response relationships to noradrenaline (NA) reveal increased resistance even at maximal dilatation, a steeper "resistance curve" and lowered maximal pressor response in SHR, but no difference in NA threshold compared to normotensive control rats (NCR). Furthermore, aortic strips and portal veins from SHR and NCR did not differ concerning NA sensitivity. The SHR and NCR resistance curves are virtually identical with those of model resistance vessels, assumed to differ only in wall/lumen ratios (cf Folkow *et al.* 1973).

Adding another vasoactive agent, 5-hydroxytryptamine (5-HT), which in some circumstances may contribute to ordinary vascular control, Häusser and Finch (1972) observed only minor responses in hindquarter vascular beds and no real difference between SHR and NCR during the mentioned resistance curve characteristics. According to Häusser and Finch these findings might challenge the concept that an altered resistance vessel design is of hemodynamic importance in hypertension. In perfused mesenteric arteries, on the other hand, they find an increased sensitivity and reactivity to 5-HT in SHR, while isolated renal arteries are less responsive in SHR than in NCR. It should here be stressed that 5-HT exerts more complex effects than NA, constricting proximal resistance vessels (like the mesenteric artery) while dilating more distal ones, at least in some circuits (cf Merrill *et al.* 1974). Further, a naturally increased wall/lumen ratio may well be associated with independent changes in smooth muscle responsiveness to vasoactive agents. The resistance curves are then, of course, influenced by at least two separate factors, but they can nevertheless often be experimentally distinguished (Folkow 1976). For such reasons it was considered of interest to further compare vascular smooth muscle responsiveness to 5-HT in SHR and NCR on two widely utilized proximal parts of the systemic circuit and on the hemodynamically all-important microvessels.

In paired, constant-flow perfusions of isolated hindquarter vascular beds from adult (6-8 months) male SHR and NCR (with mean arterial pressures of 130 ± 9 and 107 ± 2 mmHg, respectively) the pressor response to increasing 5-HT concentrations were explored from maximal dilatation to maximal constriction (cf Folkow *et al.* 1970). Once the responses to supramaximal 5-HT concentrations were reached, it was tested whether the resistance vessels could further constrict, by superimposing maximal doses of vasopressin

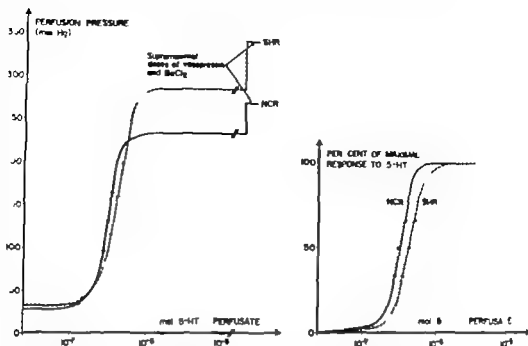


Fig. 1 Left part: Mean dose-response curves for 5-HT from isolated hindquarter vascular beds of SHR and NCR, perfused at constant flow. Right part: Mean dose-response curves for 5-HT so recalculated that the respective maximal resistance increases in NCR and SHR are both set to 100 per cent.

and barium ions. — When strips of the aorta or isolated portal veins were studied, 9 pairs of these preparations from adult matched SHR and NCR were kept in the same organ bath and thus exposed to identical increments in 5-HT concentration (cf. Hallböök, Lundgren and Weiss 1971).

With respect to the 5-HT responsiveness of isolated aortic strips and portal veins from SHR and NCR both SHR preparations exhibited supersensitivity, particularly the aortic strips. Thus, for SHR aortic smooth muscle ED_{50} was $1.4 \pm 0.1 \cdot 10^{-6}$ M compared with $1.4 \pm 0.3 \cdot 10^{-6}$ M for NCR ($p < 0.01$). For portal vein the 5-HT sensitivity differed less between SHR and NCR, ED_{50} being $1.0 \pm 0.2 \cdot 10^{-6}$ M and $1.6 \pm 0.2 \cdot 10^{-6}$ M respectively ($p = 0.03$).

Concerning the hindquarter resistance vessels the situation was quite different, as shown in Fig. 1. Evidently impressive resistance increases are induced by 5-HT both in the NCR and SHR hindquarter resistance vessels (left part). Further, despite the higher distending pressure the SHR vessels clearly respond to 5-HT with a more extensive luminal narrowing than the NCR ones, as is the case also for NA vasopressin or barium ions, which latter two agents were given also here once the maximum 5-HT response was reached. In the right part, which better reveals a possible difference in 5-HT sensitivity between SHR and NCR, there is a parallel shift to the right of the SHR resistance curve, revealing a reduced 5-HT sensitivity in SHR ($p < 0.001$).

One difference from e.g. NA responses is that the SHR and NCR resistance curves to 5-HT hardly differ in steepness, despite far higher maximal pressor responses in SHR ($p = 0.001$). This may (Fig. 1 left part) be due to the lower 5-HT sensitivity in SHR (Fig. 1 right part), and/or the most distal hindquarter vessels may be poorly if at all constricted by 5-HT as observed in other species (Merrill *et al.* 1974). In addition, the 5-HT receptors may have a different distribution in the concentric muscle layers than the NA ones (Krishnamurthy 1971) or some

not affecting their relative contributions to luminal narrowing and hence to the curve shape and its dependence on wall thickness. The NA influence is less complex since the adrenergic neuroeffector junctions, and probably most α -receptors have an adventitial location, from which neurogenic contractions are initiated, presumably engaging inner layers solely by myogenic propagation (cf. Ljung, Bevan and Su 1973).

In any case, it is clear first that 5-HT induces far stronger vasoconstrictor responses in the portal arteries than reported by Haeusler and Finch (1972), second, that the SHR resistance vessels regularly constrict more powerfully to high 5-HT concentrations than the NCR ones. However, independent of this resistance vessel "hyperreactivity" in SHR, the corresponding smooth muscles are, if anything, less sensitive to 5-HT than the NCR ones.

In conclusion, the situation concerning vascular smooth muscle sensitivity to 5-HT in SHR and NCR is quite complex. While aortic and portal vein smooth muscle (like mesenteric arteries) are more sensitive to 5-HT in SHR than in NCR, those of the true resistance vessels are less sensitive, though they nevertheless display the characteristic hyperreactivity that flows from an altered design. The present results thus illustrate that findings concerning smooth muscle responsiveness should not be generalized from one vascular preparation to the other further than a structurally based "hyperreactivity" of SHR resistance vessels may well be separated from independent shifts in smooth muscle sensitivity. Therefore, the presence of various combinations of vascular design and effector responsiveness, with differences even between various cardiovascular regions, should always be borne in mind, particularly in studies of hypertensive situations. In any case, the 5-HT supersensitivity in proximal vessels is hardly of any direct relevance for the SHR hypertension, because the hemodynamically more important distal vessels exhibit reduced sensitivity to 5-HT. The possibility remains that these apparently bizarre shifts in sensitivity indirectly reflect an alteration of some basic effector characteristic in SHR, which in other respects may be of considerable importance.

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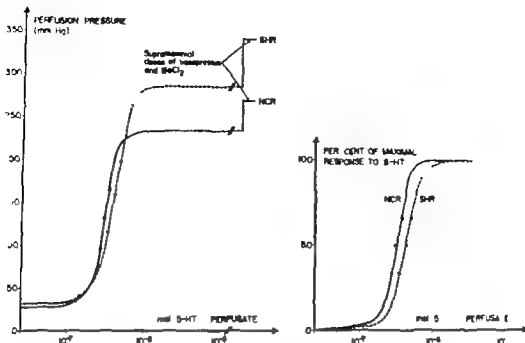


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Ca⁺⁺ Sensitive Microelectrode: Intracellular Steady State Measurement in Nerve Cell

By

G. R. J. CHRISTOFFERSEN and LEIF SIMONSEN

A Ca⁺⁺ sensitive microelectrode of detection limit below intracellular activities was developed by Christoffersen and Johansen (1976) and shortly after by Brown *et al* (1976). Electrodes having tip-sizes down to 1 μ m are now tested in neural somata of *Helix pomatia*. A steady state activity of $4.5 \cdot 10^{-7}$ M is concluded from the measurement.

The electrode was prepared as described by Christoffersen and Johansen (1976) except for two changes. The PVC content was 10% instead of 40% and the external tip diameter was 1 μ m instead of 20 μ m.

The electrode was calibrated against a calomel electrode in solutions of the same ionic strength as that of the Ringer solution (0.125). Solutions of pCa values 1.96 to 4.51 were NaCl-CaCl₂ mixtures, single ion activity coefficient were calculated according to Butler (1968). Solutions of pCa values from 4.30 to 9.61 were made as Ca-EGTA buffers using 5 mM CaCl₂, 10 mM Na EGTA and adjusting pH with Tris-maleate buffer. Concentration of Ca⁺⁺ was calculated as the reciprocal $K_{Ca-EGTA}$ in Caldwell (1970) and the Ca⁺⁺ activity-coefficient was chosen equal to that of the CaCl₂-NaCl mixtures having the lowest Ca content.

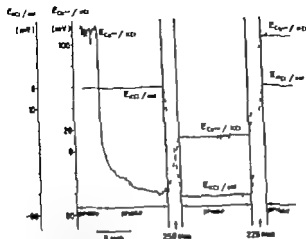
The potential difference ($E_{Ca/cal}$) vs pCa, was rectilinear in the pCa range 1.96 to 11.0 (corresponding to the range +71 mV to -98 mV) with a slope α of 28.0 mV/pCa (23°C).

The Ringer solution was that described by Kerkut and Thomas (1965). It has a calcium concentration of 7 mM. $E_{Ca/cal}$ in the Ringer was 51 mV corresponding to a Ca⁺⁺ activity of 2.0 mM.

3 electrodes were used for recording intracellular Ca-activity: 1) A calomel electrode permanently placed in the bathing solution, 2) the Ca⁺⁺-sensitive electrode and 3) a glass microelectrode filled with 2.5 M KCl. Potential difference recordings were made between 2 and 3 ($E_{Ca/KCl}$) and between 3 and 1 ($E_{KCl/cal}$).

The cell was the large medio-rostral cell in the right parietal ganglion.

The results from the experiment is shown in Fig. 1. In phase 1 both microelectrodes were placed in the Ringer solution, and $E_{KCl/cal}$ was adjusted to zero. At phase 2 the Ca⁺⁺ sensitive electrode impaled the cell. At phase 3 the KCl-filled electrode also impaled the cell, subtracting the cell membrane potential -53 mV from the $E_{Ca/KCl}$ level of phase 2, and at phase 4 both microelectrodes were withdrawn from the cell. The high noise level of the



recording of the potential difference, $E_{\text{Ca}^{++}/\text{KCl}}$, between Ca^{++} sensitive microelectrode and microelectrode, and the potential difference, $E_{\text{KCl}/\text{mem}}$, between the KCl-filled microelectrode and electrode (placed in the Ringer solution) the latter potential was counterbalanced to zero electrodes were in the Ringer solution.

recording in phase 1 resulted from attempts to penetrate the cell membrane. In $\text{Ca}^{++}/\text{KCl}$ was constant within 6 mV for 8 h, and the $E_{\text{Ca}^{++}/\text{KCl}}$ difference between phase 4 was 10 mV.

Ion of intracellular Ca^{++} activity

$$\text{Ca}^{++}/\text{KCl} \quad (E_{\text{Ca}^{++}/\text{KCl}}) = \log \frac{a_{\text{Ca}^{++}} K_{\text{Ca}^{++}/\text{KCl}} a_{\text{K}^{+}}}{a_{\text{K}^{+}} K_{\text{Ca}^{++}/\text{KCl}} a_{\text{Ca}^{++}}} + \log \frac{K_{\text{Ca}^{++}/\text{KCl}} a_{\text{Ca}^{++}}}{K_{\text{Ca}^{++}/\text{KCl}} a_{\text{Ca}^{++}}}$$

Walker (1971). $K_{\text{Ca}^{++}}$ is the selectivity coefficient for the electroactive membrane i.e. $a_{\text{Ca}^{++}}$ and $a_{\text{K}^{+}}$ are extracellular and intracellular activities of these ions. x is the calibration plot.

Activity coefficients were obtained in the following way. At $\text{pCa} = 7.6$ (activity) $K_{\text{Ca}^{++}/\text{KCl}}$ and $K_{\text{Ca}^{++}/\text{Na}^{+}}$ were sought by adding 0.1 M (ionic strength) MgCl_2 , KCl or NaCl to the buffer. The resulting positive change of potential, being a sum of the response at large sites in the electroactive membrane from the interfering ion species and an change in ionic strength, was corrected for the latter by adding a supposedly non-interfering ion choline (0.1 M choline chloride) to an EGTA-buffer ($\text{pCa} = 7.6$). Addition of the direct intracellular contribution from Mg to $(E_{\text{Ca}^{++}/\text{KCl}})_0$ (phase 3) was measured as presumed intracellular activity of Mg (1 mM) to an EGTA-buffer of $\text{pCa} = 7.6$. Intracellular pCa was preliminarily approximated intracellular pCa obtained directly from $(E_{\text{Ca}^{++}/\text{KCl}})_0$ (phase 1), using the calibration plot disregarding interference from foreign ions ($\text{pCa} = 6.3$). Changes of potential with addition of interfering ions are listed in Table I with calculated activity coefficients obtained from.

$$(E_{\text{Ca}^{++}/\text{KCl}})_{\text{corrected}} = \log \frac{a_{\text{Ca}^{++}} K_{\text{Ca}^{++}/\text{KCl}} a_{\text{K}^{+}}}{a_{\text{K}^{+}}}$$

TABLE I Potential values from the Ca^{++} sensitive electrode for calculation of selectivity coefficients. Interfering ion species are added to EGTA buffers, changing ionic strength by 0.1 M. Resulting changes of potential are $\Delta(E_{\text{Ca}^{++}/\text{cal}})_{\text{varied}}$. Effect of ionic strength is corrected for ($\Delta(E_{\text{Ca}^{++}/\text{cal}})_{\text{constant}}$) by subtracting $\Delta(E_{\text{Ca}^{++}/\text{cal}})_{\text{varied}}$ for 0.1 M choline from the same parameter for other ion species. All figures are mean of 3 expts. Mean variation between expts. was 32 mV for a given parameter

Ion added to EGTA Buffer	$\Delta(E_{\text{Ca}^{++}/\text{cal}})_{\text{varied}}$ (mV)	$\Delta(E_{\text{Ca}^{++}/\text{cal}})_{\text{constant}}$ (mV)	pCa	$K_{\text{Ca},i}$
Choline (0.100 M)	8		7.6	
N ⁺ (0.100 M)	17	9	7.6	$33 \cdot 10^{-4}$
K ⁺ (0.100 M)	14	6	7.6	$17 \cdot 10^{-4}$
Mg ⁺⁺ (0.033 M)	51	43	7.6	$3 \cdot 10^{-3}$
Mg ⁺⁺ (0.001 M)		2	6.3	$5 \cdot 10^{-4}$

When values of $a_{\text{Ca}} = 2 \text{ mM}$, $a_{\text{Ca}} = 5 \cdot 10^{-4} \text{ M}$ (pCa = 6.3), $a_{\text{K}} = 4 \text{ mM}$ (Thomas, 1977), $a_{\text{K}} = 68 \text{ mM}$ (measured with a K⁺ selective electrode) and extracellular values of other ions from the Ringer are inserted in eq. 1 ii reduces to the following significant terms.

$$(E_{\text{Ca}^{++}/\text{cal}})_0 - (E_{\text{Ca}^{++}/\text{cal}})_1 = \alpha \log \frac{a_{\text{Ca}}}{a_{\text{Ca}} + a_{\text{Li,Na}} + a_{\text{K}}^2 K_{\text{Ca,K}}}$$

Setting $(E_{\text{Ca}^{++}/\text{cal}})_0 - (E_{\text{Ca}^{++}/\text{cal}})_1$ equal to the difference between the level of phase 3 and the mean of the level of phase 1 and phase 4 (Fig. 1) a_{Ca} emerges from eqn. 3 as $4.5 \cdot 10^{-4} \text{ M}$ in 3 expts. within the linear part of the calibration curve. This value may be compared to $3 \cdot 10^{-4} \text{ M}$ in squid axons derived from aequorin fluorescence (Baker 1972). The behavior of the electrode in non-steady-state situations is being investigated.

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The Intracellular Concentration of 2,3-Diphosphoglycerate in Type II A Hyperlipoproteinemia

By

A. LEHTONEN, J. VUOKARI and A. KARI

It has been proposed, that hypoxia is a major feature of arteriosclerosis (Hueper 1944), and that an increased erythrocyte oxygen affinity is a prominent feature in the production of arterial wall hypoxia (Brewer 1972). Rabbits on an atherogenic diet exposed to hypoxia have higher cholesterol levels than control rabbits on an atherogenic diet (Kjeldsen *et al.* 1968). Kjeldsen (1969) has reported a positive correlation between carboxyhemoglobin levels and serum cholesterol levels in smokers. The increased risk of arteriosclerotic disease in cigarette smokers is well-established. The importance of 2,3-diphosphoglycerate for the oxygen unloading properties of hemoglobin is well known (Chastain and Curdick 1967). The overall profile of transmural oxygen tension in experimental atherosclerosis was markedly different from normal (Heughan *et al.* 1973). The purpose of this study was to investigate the effect of hemoglobin and 2,3-diphosphoglycerate concentration of red blood cells in patients of type II A hyperlipoproteinemia.

1 patient had primary type II A hyperlipoproteinemia. The patients are kept on proper diet for at least 2 months, and those with cholesterol values below 7.5 mmol/l are excluded. All patients were clinically stable (3 females, 5 males, aged 33-55 years). Two men are smokers. The venous blood samples were taken after overnight fasting for determination of cholesterol, triglycerides, and phosphorus. Plasma low density lipoprotein (LDL) isolation was carried out by density gradient centrifugation method (Vikari *et al.* 1974).

The oxygen affinity of the blood was determined by equilibrating the blood sample with gas mixtures of known composition and previously determined partial pressures of oxygen (26.3 mmHg) and carbon dioxide (40 mmHg). The oxygen-hemoglobin saturation of the sample was then measured spectrophotometrically according to Siggaard-Andersen *et al.* (1962). The oxygen affinity of the blood is expressed as standard P_{50} (the oxygen tension giving 50% oxygen-hemoglobin saturation at pCO_2 40.0 mmHg and pH 7.40) and was calculated by means of Hill's equation (Rorth *et al.* 1972) and corrected to pH 7.40 according to Drury *et al.* (1963).

The 2,3-diphosphoglycerate (DPG) concentration of red blood cells was determined according to Kow and Altmann (1970) and expressed as micromoles per liter of packed red blood cells. The values of intracellular 2,3-diphosphoglycerate concentration and standard P_{50} obtained from healthy women (aged 25-49, mean 31.9 years) and men (aged 22-55, mean 35.5 years) were used as controls.

The mean results of the study are shown in Table I. No significant differences in the standard P_{50} were found. The DPG concentrations of the red blood cells of women were higher than the mean values of the controls ($p < 0.01$), but the DPG concentrations of the red blood cells of men were unchanged. Two women with the highest total and LDL-cholesterol concentra-

TABLE I Serum cholesterol and 2,3-DPG in type II A hyperlipoproteinaemia

The patients had II A lipoprotein pattern on cellulose acetate electrophoresis. They were kept on hypocholesterolemic diet for at least 3 months before taking blood samples. 2,3-DPG of the female group differs significantly ($p < 0.01$) from the controls.

Initials	Sex	Age	Smok g	Serum cholesterol mmol/l	2,3-DPG mmol/l	Hemoglobin g/l	Phosphorus mmol/l	P_{50} mmHg
L. E.	♂	45	+(-)	11.9	4.9	138.3	1.1	25.2
L. M.	♂	28	-	11.6	4.6	148.7	0.9	25.2
H. K.	♂	32	-	9.1	4.4	152.6	0.5	24.9
L. T.	♂	4	+(-)	9.1	4.4	123.2	1.0	26.8
S. O.	♂	22	-	9.1	4.4	132.8	0.9	22.7
♂	30.2	2-45		10.2 ± 1.5	4.5 ± 0.2	139.1 ± 11.9	0.88 ± 0.23	25.0 ± 1.5
♂	33.5	35-55		Controls (n=10)	4.7 ± 0.4			26.4 ± 1.4
T. L.	♀	49	-	13.4	5.8	101.7	1.2	23.8
S. M.	♀	55	-	12.6	6.6	110.2	1.3	25.3
K. L.	♀	41	-	8.7	4.9	132.7	0.9	26.5
S. R.	♀	37	-	8.5	5.0	131.7	0.8	23.8
H. E.	♀	37	-	7.5	5.2	150.8	1.2	28.6
♀	43.8	37-55		10.1 ± 2.7	$5.5 \pm 0.7^*$	125.4 ± 19.6	1.08 ± 0.22	26.0 ± 1.8
♀	31.9	26-49		Controls (n=10)	4.6 ± 0.4			23.4 ± 1.4

tions had also the highest concentrations of DPG. Plasma calcium and phosphorus concentrations were normal in all patients (except one low phosphorus concentration).

DPG exerts an effect on the hemoglobin dissociation curve similar to that of CO. Increasing concentrations of DPG shift the P_{50} to the right. It has been postulated, that hypoxia is important in the production of arteriosclerosis (Hueper 1944). Prolonged tissue hypoxia has also been suspected in the development of diabetic microangiopathy (Ashton and Path 1966). Several clinical conditions such as anemia (Torrance *et al.* 1970) and low output cardiac disease (Woodson *et al.* 1970) have been detected where compensatory changes in the 2,3-DPG level of the red blood cell occur.

No significant differences were found in the erythrocyte 2,3-DPG concentration between normal and diabetic patients, but in the diabetic patients, there could be demonstrated significant fluctuations of DPG concentrations as determined by the metabolic control of their diabetes (Standl and Kolb 1973). Diabetics with pronounced type I or type V hyperlipemia showed markedly increased hemoglobin-oxygen affinity (Ditzel and Dyerberg 1977). Theoretically an increase in 2,3-DPG should increase oxygen release in the peripheral tissue at any given partial pressure of oxygen. It still remains open to question whether the ability of women to increase the concentration of DPG in fact protects them against hypoxia in the arterial wall and thus the development of atherosclerosis.

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Innervation of the Duodenal Submucosal Brunner's Glands

By

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Innervation of the gastrointestinal smooth muscle has been studied extensively using modern specific neurohistochemical and electron microscopical techniques (for ref. see e.g. Kyösola and Rechartt 1974). At the same time, very little is known about the innervation of the duodenal submucosal Brunner's glands. The present study was undertaken in order to partly fill this gap in knowledge.

Small tissue pieces from the submucosal layer of the duodenum of the cat were excised under general anesthesia induced by i.p. injection of sodium thiopentone and immediately processed for 1) fluorescence microscopy using two fluorescence histochemical methods: 1) the formaldehyde-induced fluorescence histochemical method (Eränkö 1967 a), 2) the glyoxylic acid-induced fluorescence histochemical method (Lindvall and Björklund 1974).

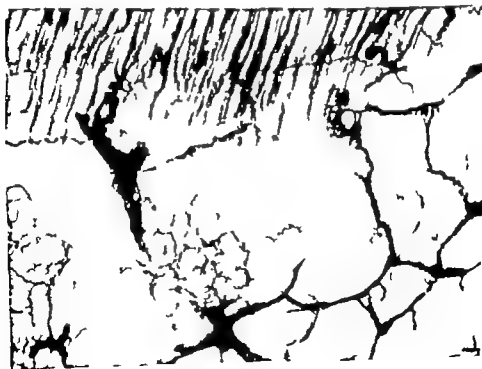
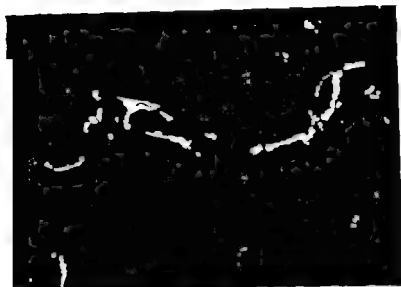


Fig. 1. Delicate small-meshed nerve nets consisting of terminal varicose ramifications of acetylcholinesterase-positive axons enclose the Brunner's glands in "basket-like" fashion, an arrangement suggestive of functional cholinergic (vagal) innervation.



wide distribution of fluorescent (adrenergic) axons in the submucosal layer of the duodenum, close to the Brunner's glands

I et al. 1974), and II) for the demonstration of acetylcholinesterase-positive nerves in sections using two thiocholine techniques: 1) Gomori's modification (1952) of the Friderwald technique (1949), 2) the direct-coloring copper-ferrocyanide introduced by Karnovsky and Roots (1964).

Small-meshed nerve nets consisting of terminal varicose ramifications of acetylcholinesterase-positive axons were seen to enclose the Brunner's glands in basket-like arrangement suggesting of functional innervation (Fig. 1). In contrast, the distribution of fluorescent axons in the submucosal layer of the duodenum was relatively scarce, and it was impossible to demonstrate a specific functional adrenergic innervation of the Brunner's glands (see Fig. 2).

The acetylcholinesterase activity can be considered to be a tentative suggestion of the cholinergic nature of the neuron (Erlanko 1967 b). Thus, the acetylcholinesterase positive fibers apposing onto the Brunner's glands obviously represent "truly cholinergic" elements, instead of being only cholinceptive. This concept is further corroborated by the observation that only very few adrenergic axons were seen in the immediate vicinity of the Brunner's glands. The conclusion thus seems justified that the neural regulation of the secretory activity of the Brunner's glands is (at least mainly) cholinergic, and consequently of vagal origin.

Zusammenfassung: Die Innervation der duodenalen submucösen Drüsen Brunners der Ratte wurde mit zwei Fluoreszenzmethoden zur Sichtbarmachung von Monoaminen und mit histochemischen Methoden zum Nachweis von acetylcholinesterase-positiven Nerven in Gewebeschritten untersucht. Die cholinerge Innervation der Brunnerschen Drüsen ist reichlich während keine funktionelle adrenerge Innervation festgestellt werden konnte.

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Rapid and Repetitive Measurements of Blood Flow and Oxygen Consumption in the Rat Brain Using Intraarterial Xenon Injection

By

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A method for rapid and repetitive measurements of cerebral blood flow (CBF) and cerebral metabolic rate for oxygen ($CMRO_2$) in rat in situations of non-steady state, e.g. seizures, has recently been described. We have therefore applied the intra-arterial xenon injection method (Smith-Rasmussen *et al.* 1966) to the rat.

The main problem in animal studies with this technique, which has been extensively used previously, is to avoid the influence of contamination from extracerebral tissues on the xenon clearance curves. Such a contamination will result in too low flow values due to the slower clearance of extracerebral tissues. In a previous study of blood-brain-barrier permeability (Smith-Rasmussen *et al.* 1976) we described an animal preparation in which extracerebral contamination is minimal (Hertz and Bolwig 1976) and we therefore proceeded to use it for measurement of CBF and $CMRO_2$.

1-400 g Wistar rats were anaesthetized with 1.2 g/kg halothane in gas mixture of 70% N_2O and 30% O_2 . All extracerebral branches from both carotid arteries are ligated as described in detail by Hertz and Bolwig (1976). The right external carotid artery is cannulated in retrograde fashion with the tip of the cannula at the carotid bifurcation allowing the bolus to be injected into the internal carotid artery without obstruction of the artery. Cerebral venous blood from the confluence of sigmoid sinus was sampled from a catheter placed in a heart base or the heparinized process.

Mean arterial blood pressure (MABP) and rectal temperature were monitored throughout the study. A decrease in MABP by more than 20% of the initial value was avoided with blood transfusions. Blood sampled through catheter in femoral artery was used for determination of pH, $PaCO_2$ and PaO_2 . The arterial pressure was kept above 100 mmHg. After the surgical procedure halothane was switched off and the animal was paralyzed with succinyl choline 0.1 mg/kg and ventilated on a respirator 20 min elapsed before the measurements.

For determination of CBF, 50 μ l bolus of the γ -emitting isotope ^{133}Xe was injected into the internal carotid artery and the xenon was followed by external detection with a collimated NaI(Tl) crystal (specimen 1 mm) placed on the head between eye and ear on the side ipsilateral to the injection site. By means of a lead plate detection of activity in tracheostomy tube and carotid catheter was avoided.

For $CMRO_2$ determination arterial and cerebral venous blood was sampled 30 s after bolus injection. The venous blood gas tensions and total oxygen content (Bergström *et al.* 1974) were measured. $CMRO_2$ was calculated from the arterial slope (Olsson *et al.* 1971) of a semilog plot of the clearance curve using approximately the first 15 s of the curve. $CMRO_2$ was calculated by multiplying CBF with arterio-venous difference in oxygen content.

Hypocapnia was induced by adding 1-5 l/min

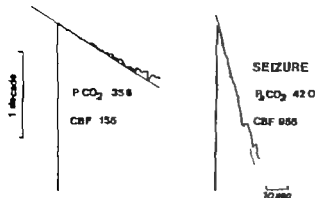


Fig. 1 Semilogarithmically recorded xenon wash-out curves. P_aCO_2 in mmHg; CBF in ml/100 g min.

Fig. 1 shows representative ^{133}Xe clearance curves in normocapnia and during seizure. Fig. 2 shows CBF values as a function of P_aCO_2 in 43 measurements in 20 rats. CMRO in 15 animals with P_aCO_2 between 22 and 42 mmHg was 10.6 ± 0.7 ml/100 g min (mean \pm S.E.).

The CBF and CMRO values found are in good agreement with the results of Eklöf *et al* (1973). It is possible to get reproducible results with the method and even very high CBF values can be measured.

Several methods for measurement of CBF in the rat are available. The most extensively studied method is the Kety-Schmidt method used by Eklöf *et al* (1973) and later by Gjedde *et al* (1975). This method is based on determination of arteriovenous differences for xenon during desaturation following inhalation of the inert gas and requires steady state conditions during 10 min.

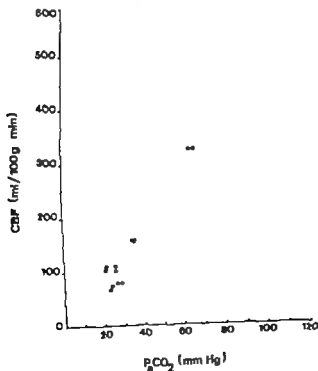


Fig. 2 CBF was obtained at different carbon dioxide tensions in the arterial blood.

uptake methods are fraught with difficulties. The antipyrine method (Reivich *et al.* 1975) is based on the incorrect assumption that antipyrine freely crosses the blood-brain barrier. In high flow situations CBF is underestimated. In the xenon uptake study (Moto *et al.* 1975) it is difficult to avoid evaporation of tracers from the tissue during arterial injection of inert gas has previously been tried by Chapman *et al.* 1975 but their results showed persistently low CBF values; the authors disregarded the risk of extracerebral contamination. In the present preparation extracerebral contamination was minimized by extensive ligation of the extracerebral branches from the carotid arteries and this is a prerequisite for the injection technique in animals. Furthermore, using the inert xenon gas there are no problems with incomplete diffusion exchange between blood and brain as with antialcohol or tritiated water. The i.a. injection method offers several advantages. It requires steady state for more than 20–30 s during which the outwash curve is obtained. It allows repeated measurements in the same animal. The method determines mainly the fast component of CBF which largely corresponds to grey matter flow. Similarly the differences in oxygen content obtained using blood from the confluence of sinuses are mainly representative for cortical structures. Both CBF and CMRO₂ values obtained with this method are slightly higher than the brain values (Nelson 1974) which must include more of the slowly perfused sparse matter with lower metabolism.

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Inhibition of Bradykinin Induced Macromolecular Leakage from Post-Capillary Venules by a β_2 -Adrenoreceptor Stimulant, Terbutaline

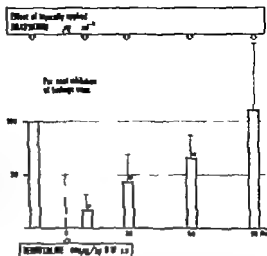
By

E. SVENJÖ, C. G. A. PERSSON and G. RUTIL

Histamine and bradykinin both increase microvascular permeability to proteins. Majno and Palade (1961) used carbon particles to study site and size of macromolecular leakage and found by electron microscopy that this increase occurred in venules of 20-30 μ m diameter and suggested that this was due to the formation of "gaps" resulting from the contraction of endothelial cells because of stimulation by histamine and bradykinin. We obtained similar results in a study based on intravital microscope observations of the extravasation of intravenously injected fluorescein-labelled macromolecules (Svenjö *et al.* (1973). Further more, using the technique of Simionescu *et al.* (1971) to demonstrate polysaccharides, we have found by electron microscopy that macromolecular leakage occurs through gaps between endothelial cells following topical application of bradykinin (Hultström and Svenjö 1976). The precise mechanism by which bradykinin and histamine induce contraction of endothelial cells is not known. The presence of contractile elements in these cells similar to those in smooth muscle suggests a contractile mechanism analogous to that of smooth muscle cells (Becker and Nachmann 1973). If this assumption is valid, it might be possible to counteract histamine and bradykinin induced endothelial cell contraction with drugs such as β -adrenoreceptor stimulants which are known to relax smooth muscle cells. Green (1972) showed that leakage of Evans Blue from vessels in the mouse peritoneum was antagonized by catecholamines and salbutamol acting predominantly on β -adrenoreceptors, and suggested that this effect was due to antagonism of endothelial cell contraction. We have used terbutaline as a β -adrenoreceptor stimulant in a study of its effect upon bradykinin-induced macromolecular leakage.

Golden hamsters weighing 70-100 g were anesthetized with pentobarbital, 9 mg/100 g b.w. i.p. A femoral vein was cannulated for supplementary injections of pentobarbital and for injection of indomethacin 2 mg/100 g b.w. Experiments were carried out on the everted hamster cheek pouch preparation as described by Duling (1973) and with our modifications (Svenjö *et al.* 1977a). This experimental model has been found suitable for quantitative intravital studies of the microvascular leakage of macromolecules such as fluorescein labelled dextran (FITC-dextran 150, $M_w = 150\,000$, Pharmacia Flow Chemicals, Uppsala, Sweden) induced by histamine and bradykinin (Svenjö *et al.* 1973). The preparation also enables simultaneous measurements of red cell velocity in single arterioles as described by Arfors *et al.* (1975). Blood flow measurements, estimated from red cell velocity and vessel diameter were relevant to estimate the effect of bradykinin and terbutaline on blood flow. In the first series of experiments 5 hamsters were used. Following i.v. injection of FITC-dextran 150 the number of FITC-dextran leakage sites in the exposed area of the

Fig. 1 Repeated topical application of bradykinin to the hamster cheek pouch preparation before and after the intravenous injection of terbutaline 50 $\mu\text{g/kg b.w.}$ Response expressed as percent of postcapillary response to bradykinin (\pm S.D.) $p < 0.05$, Student's *t*-test for paired observations.



chest pouch was between 0 and 10 sites per cm^2 (preapplication control values). Bradykinin $0.1 \mu\text{g ml}^{-1}$ was applied topically for 4 min during which the normal superfusion of the cheek pouch preparation was interrupted. The number of FITC-dextran leakage sites at postcapillary venules as counted 1 sec after the application of bradykinin had been completed. The number of microvascular leakages was maximal at the time. The rate of leakage was consequently confined to the postcapillary venule. After the extravasated FITC-dextran had been cleared from the tissue by the superfusing solution, another application of bradykinin, the time at which terbutaline ($1 \mu\text{g ml}^{-1}$) added, could be made.

The results from 5 hamsters showed that the total number of leakage sites at postcapillary venules was 88 ± 31 (S.E.) sites per cm^2 with bradykinin alone and 29 ± 11 with the combination of bradykinin and terbutaline. (Student's *t*-test for paired observations, $t = 2.803$, $P < 0.05$). These figures should be compared with the pre-application control values of 0 to 10 sites per cm^2 .

In another series of 5 hamsters the cheek pouches were exposed to bradykinin in the same way but with terbutaline given 1 min before in a dose of 50 μg per 100 g b.w. The preparation was then repeatedly exposed to a bradykinin solution ($0.1 \mu\text{g ml}^{-1}$) (Fig. 1). 10 min after the injection of terbutaline there was a significant reduction in the number of leakage sites to 17 ± 14 of the pre-injection value with bradykinin alone (Fig. 1). Repeated application of the same dose of bradykinin showed that the approximate duration of the effect of this dose of terbutaline was 1 h (Fig. 1).

Microvascular blood flow was estimated in one arteriole in each of 8 other hamsters. Bradykinin, applied as described above, increased arteriolar blood flow to 199 ± 27 and terbutaline increased it to 217 ± 20 of control blood flow. Only the bradykinin-induced increase was statistically significant (Table I).

Topical application of bradykinin resulted thus in an increase in both macromolecular leakage and blood flow. Terbutaline was found to counteract the induced increase in macromolecular leakage at postcapillary venules although terbutaline applied separately increased blood flow by 217 ± 20 ($P < 0.05$). It is unlikely that the combination of bradykinin and terbutaline should result in a smaller blood flow increase than caused by bradykinin alone.

TABLE 1 Arteriolar blood flow in ml s^{-1} . Vessel size $36.6 \pm 2.3 \mu\text{m}$ showing blood flow at start of exp. (control) and after topical application of bradykinin and terbutaline. Student's *t* test for paired observations.

	Control at start	Control immediately before	Bradykinin 0.1 $\mu\text{g ml}^{-1}$	Control immediately before	Terbutaline 1 $\mu\text{g ml}^{-1}$
n	8	8	8	6	6
x	1.47	1.04	4.04	1.02	2.21
S.D.	1.31	1.56	1.00	0.39	1.76
t	—	—	3.311	—	1.862
P	—	—	0.013	—	0.112
Blood flow in per cent of control	100		199		17

These findings suggest that the inhibition of bradykinin-induced macromolecular leakage by terbutaline would not be explained by an inhibition of the bradykinin-induced increase in blood flow but rather by a specific inhibition of the endothelial cell response of the postcapillary venules to bradykinin. After the completion of the present study further anti-leakage effects have been examined. The inhibitory effect of terbutaline on macromolecular leakage at postcapillary venules was also found with PGE₂ induced leakage although the peak blood flow following PGE₂-application was the same with as well as without terbutaline (Svensjö *et al.* 1977b). Terbutaline has been found to counteract histamine induced leakage of Evans Blue in guinea pig skin (O'Donnell and Persson 1977). Mediator induced leakage also seems to occur at gaps between venular endothelial cells in the lung (Pietra *et al.* 1971). If a direct inhibitory effect on leakage is exerted by terbutaline in the lung as well as in the cheek pouch, it might reduce mediator-induced oedema and contribute to the effectiveness of terbutaline in asthma.

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Interactions of Potassium, Noradrenaline and Acetylcholine in the Guinea Pig Vas Deferens

By

P. HEDQVIST and U. S. v. EULER

twitch response of the isolated guinea pig vas deferens to brief trains of stimuli delivered and stimulation is, as a rule, inhibited by low and enhanced by higher concentrations of noradrenaline (NA). The enhancement of the twitch response by NA is due to a post-junctional stimulant effect, which is annulled by α -adrenoceptor blockers, whereas the rise in twitch height is due to NA acting on postjunctional β_1 -adrenoceptors as well as α - and β_2 -adrenoceptors, which are presumably prejunctionally located (Aurbache and 1971 Hedqvist and Euler 1976). Since the twitch response itself is not inhibited by either α -adrenoceptor blockers it has been proposed that the twitch contraction (in contrast to the slow contraction at prolonged nerve stimulation) is not mediated by but rather elicited by NA (Aurbache and Zar 1971 Hedqvist and Euler 1976, Jenkins *et al.* 1975). The guinea pig vas deferens responds with twitch-like contractions to moderate concentrations of KCl, and it has been suggested that K ions may be involved in the twitch response to nerve stimulation (Euler and Hedqvist 1975 Sjöström 1976).

We report here some preliminary results of studies concerning interactions between potassium, NA and acetylcholine (ACh) on the guinea pig vas deferens, supporting the proposed role of K ions and possibly ACh in the twitch response.

Guinea pig vas deferens are mounted in 5 ml organ bath using Tyrode as bath fluid (for composition, Hedqvist and Euler 1976). The solution was kept at 37°C and gassed with 5% CO₂ in O₂. Transmural twitches were delivered by Grass S4 stimulator through platinum electrodes along the wall of the bath. Twitch contractions are elicited at 1 min intervals by 10-20 biphasic pulses (5-10 Hz, pulse duration 1 ms, transmural voltage), each specifically activates the nerve. In some cases the smooth muscle cells were relaxed directly by prolonging the pulse duration to 30 ms, after blockade of nerve conduction with tetrodotoxin (TTX). The contractions were recorded with an isometric transducer (Harvard heart/smooth muscle transducer) on Honeywell ink writer. The load is 0.25 g.

Addition of KCl to the bath fluid significantly and dose-dependently increased the twitch response to transmural nerve stimulation. In the higher dose range this enhancement was associated with a direct contraction of the vas deferens (Fig. 1). In the non-stimulated preparation KCl caused well reproducible, twitch-like contractions when the whole bath fluid was rapidly replaced from a special container and the organ exposed to concentrations of 3-5 times the normal (Fig. 2). A twitch response at these concentrations points to a high sensitivity of the reacting system to K ions. The KCl-induced twitch was annulled by addition of 0.04-0.06 µg/ml TTX, as were twitches elicited by nerve stimulation (*cf.* Hedqvist and Euler 1976).



Fig. 1

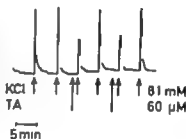


Fig. 2

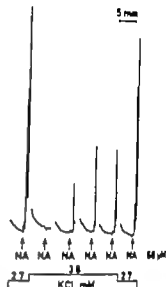


Fig. 3

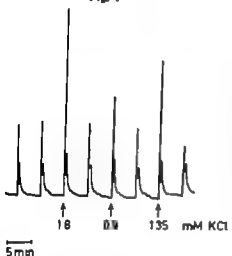


Fig. 4

Fig. 1 Effect of cumulative doses of KCl (4 mM) on twitch response to transmural nerve stimulation, 5 Hz, 1 ms, 10 pulses, at 1 min intervals in guinea pig vas deferens. Wash at dot.

Fig. 2 Effect of tyramine (TA) (60 μ M) on twitch-like contraction induced by substituting normal Tyrode by Tyrode containing 8.1 mM KCl.

Fig. 3 Inhibition of NA-induced twitch-like contraction by raising KCl concentration in bath fluid from 2.7 to 3.6 mM. Wash at dots.

Fig. 4 Twitch-like response to ACh (1.1 μ M) enhanced by simultaneous addition of KCl (0.9-1.8 mM) (arrows). Wash after each application.

It seemed of interest to study whether the KCl-induced twitch was modified by NA or NA-releasing amines in the same way as the twitch induced by nerve stimulation (cf Hedqvist and Euler 1976). This proved to be the case. Thus, tyramine (60 μ M) markedly reduced the twitch induced by Tyrode solution containing 8.1-10.8 mM KCl (Fig. 2) and a similar inhibition of the KCl twitch was obtained with NA (6-12 μ M).

It is well established that NA can produce twitch-like contractions, although the dose needed is usually high, of the order of 60 μ M or more. The twitch elicited by NA was annulled or markedly reduced by addition of 1-3 mM KCl (Fig. 3).

Compared to NA, ACh is much more prone to induce twitch contraction, and it consistently enhances the twitch induced by nerve stimulation (cf Sjöstrand and Swedin 1968). The twitch elicited by 1.1 μ M ACh was, like that of KCl, inhibited by NA (6-12 μ M). It was also observed that the ACh-induced twitch is highly sensitive to potassium. Thus, less than

mM KCl added simultaneously with 1.1 μ M ACh strongly enhanced the response (Fig. 4) as the response elicited by ACh was still obtainable after TTX, suggesting a direct response of the muscle cells (*cf.* Goodman and Weiss 1971).

The observation that TTX annulled the twitch induced by KCl indicates that KCl in the concentrations used (8.1–10.8 mM) is unable to activate the muscle cell directly in contrast to ACh or electrical stimulation at increased pulse duration (*cf.* Goodman and Weiss 1971). It may thus be assumed that KCl in these concentrations normally elicits the twitch by polarizing either the nerve terminal, allowing transmission, or the specialized postjunctional area of the muscle membrane, or both. The inability of KCl to evoke a twitch after TX may therefore be due either to a prejunctional or a postjunctional membrane block, which might reduce, but not prevent, the action of ACh or electrical stimulation with pulses of extended duration, assuming that the latter effects are mainly extrajunctional. The annulment by TTX of the twitch response to brief electrical pulses and to low concentrations of KCl may therefore in both cases be due to a blockade of the action of potassium, on the assumption that potassium is a prerequisite for triggering the nerve-induced twitch.

It has been shown that relatively high concentrations of KCl, of the order of 20 mM strongly increases the ability of NA to produce a twitch (Stjärne 1976). The present observations that approximately 5 times lower concentrations of KCl are sufficient to strongly potentiate the effect of ACh, that even lower KCl concentrations suffice to annul the NA-induced twitch, and that also NA may inhibit the KCl-induced twitch point to an intricate relationship between NA, potassium and ACh and to a pivotal role of K⁺ ions in the twitch transmission. The strong enhancement of the twitch response to ACh by low concentrations of KCl is of interest also in view of the postulated participation of ACh in the twitch response to nerve stimulation (*cf.* Birmingham 1966). An interaction of ACh with K⁺ ions during the nerve stimulation appears a possibility.

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frequently found close to blood vessels (Olgart *et al* 1977). Antidromic stimulation of the inferior alveolar nerve regularly increases blood flow in the dental pulp, an effect that is not influenced by atropine or blockade with β -receptor antagonists (Gazelius and Olgart to be published). The increase of SPLI in the pulp superfusates during such stimulation can be taken as an evidence that SP is released and takes part in the vascular response. SP is following the suggested release and after having exerted its effect diffuse into the interstitial fluid rather than being directly eliminated into the blood stream. This may contribute to the explanation why in a previous study no elevation of SPLI levels were noted in bio samples collected following antidromic nerve stimulation of nerves to the tongue, skin and nose of dogs and cats (Burcher *et al* 1977).

In summary the present study supports previous claims for the existence of SPLI in the dental pulp and demonstrates the release of SPLI from this tissue. This observation may support the hypothesis presented by Lembeck (1977) that SP is involved in the axon reflex mechanism that was originally described by Dale (1935). If so SP may play a role for the development of the inflammatory response associated with injury and pain in peripheral tissues.

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Interactions of Potassium, Noradrenaline and Acetylcholine in the Guinea Pig Vas Deferens

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Release of Substance P-like Immunoreactivity from the Dental Pulp

Supplement appended.

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Units	Symbols
kilogramme	kg
second, millisecond	s ms
mole, millimole, micromole, nanomole, picomole	mol mmol μ mol nmol pmol
meter millimeter	m mm
micrometer	μ m
nanometer	nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N (kg m/s^2)
pascal (pressure)	Pa (N/ m^2)
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/ m^2)

Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa)
curie	Cl
liter milliliter microliter	l ml μ l
degree Celsius	$^{\circ}$ C

Conversion factors to be given in Methods.

